SORTING SIGNALS OF *BORRELIA* LIPOPROTEINS

BY

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Submitted to the graduate degree program in Microbiology,
Molecular Genetics and Immunology and the
Graduate Faculty of the University of
Kansas in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy.

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February 17, 2009

Acknowledgements

I cannot emphasize enough the role that my advisor, Wolf Zückert, has played in my development as a graduate student. It is with his patient help and guidance that I am even in a position to receive this degree. Taking me on as his first student was sure to be a high-risk endeavor, but he always welcomed my input whether he secretly wanted it or not. More than a mentor, he is and will always be a close friend. I can say with certainty that the high of my graduate career came in early 2006 when Wolf elatedly gave me the news that he had received his first major research grant. I hope to replicate that feeling myself one day and I hope that the work I have done in his lab may in some small way contribute to his certain future achievements as a researcher.

I am humbled by the amount of support given to me by all faculty, staff, and students in the Microbiology department at KUMC. Everyone in the Zückert lab has been a great friend and co-worker. I wish Oz, Kristina, Christine, Shiyong, and Min success in their future careers and hope to maintain close friendships as we inevitably scatter across the world in the coming years. I also appreciate their refreshing honesty in telling me when I was wrong about something, a regular event and pastime in the lab! I would like to give special thanks to Shiyong Chen for his helpful assistance with the His-tag pulldowns and MudPIT analyses. Sebastien Pichoff and Christina Hester of the Lutkenhaus lab provided all kinds of encouraging words and advice throughout my time at KUMC, with Christina being especially helpful during my writing phase.

My committee members have all been integral to my growth as a researcher. Bill Picking was my undergraduate microbiology/bacterial pathogenesis instructor at KU and introduced me to Wolf in 2003. Joe Lutkenhaus' helpful advice and comments were unmatched, and his ability to recall any and all facts is the stuff of legends. I always enjoyed our long discussions in the hallway. Kee-Jun Kim's support and advice on my comprehensive and final exam committees was especially helpful, and Mark Fisher's chats on protein folding while riding bikes in rural Missouri were always entertaining.

I would like to thank A. Keith Dunker (IUPUI) for his helpful assistance with the protein disorder predictions and Ian Henderson (University of Birmingham, UK), Tony Pugsley (Institut Pasteur), Hajime Tokuda (University of Tokyo), Brian Stevenson (University of Kentucky), Patti Rosa (Rocky Mountain Labs), and Phil Stewart (Rocky Mountain Labs) for their thoughts on lipoprotein transport. Selene Swanson of the Stowers Institute was critical to helping us set up the MudPIT experiments and explaining the volumes of data to us. Roger Tsien (UCSD) and Patrick Viollier (Case Western) provided us with the mRFP1 we use throughout these studies. I would also like to thank the KUMC Biomedical Research Training Program for funding during my final two years of graduate work.

Finally, I need to thank my parents, Don and Susan, who have been incredibly supportive of my career, even if they weren't entirely sure what it was I have been

doing over the past six years! I will be forever indebted to their constant encouragement and help along the way. The same is true of my sisters, Lindsay and Lara, and their husbands, Justin and Mike. I am proud of everything everyone in my family has accomplished in their lives and pray that I can achieve half the successes they have enjoyed. To my extended family, aunts, uncles, and grandparents: I love you all. I would also like thank my girlfriend, Autumn, for her patience, kindness, and love as I finished up my dissertation. I hope that I can provide you with the same generosity you provided me as you embark on the last legs of your own graduate work.

This dissertation is dedicated to my grandfather, Fred Markowitz. From my early childhood, he provided me with the inspiration to climb as high as I could up the educational ladder. He received his Ph.D. in English from the University of Kansas and was one of the reasons I chose to do my undergraduate work in Lawrence. I told him once that I wanted to be like him and that I would also "grow up to get *my* Ph.D." To be honest, I was seven years old at the time and had no idea what a "Ph.D." even was. But twenty-two years later, grandpa, here it is – I did it.

Table of contents

List of Figures

List of Tables

Abbreviations

CL	cardiolipin
CM	cytoplasmic (inner) membrane
EM	erythema migrans
IM	inner membrane
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
ORF	open reading frame
PC	protoplasmic cylinder, phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PPM	polyprenol monophosphomannose
SOE	sequence overlap extension

Abstract

Bacterial lipoproteins play important roles in prokaryotic physiology, protein transport, membrane biogenesis, and pathogenesis. Produced in the cytoplasm, these proteins are N-terminally modified by the covalent addition of a fatty-acid moiety onto a conserved cysteine residue. This acylation step allows for anchoring of the protein into a lipid bilayer in the absence of membrane-spanning domains. Lipoproteins of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, are of particular interest because of their presence on the bacterial cell surface at the interface between pathogen and potential host. In *Borrelia*, lipoproteins must cross both an inner and outer membrane en route to the cell surface. In *Escherichia coli*, the process of transporting lipoproteins between two membranes has been extensively studied, but occurs only within the boundaries of the periplasmic space. The mechanistic underpinnings of this process in *Borrelia* are not understood. Here, we provide the first insights into lipoprotein transport to the surface of the *B. burgdorferi* cell.

Using fusions of a red fluorescent reporter protein to the *B. burgdorferi* surface lipoprotein OspA, we show that the sorting rules of *E. coli* do not apply but that information encoded within the first five residues of the mature lipoprotein is capable of directing proteins to the *Borrelia* cell surface. Negatively-charged residues within a certain context can result in the retention of a lipoprotein in the inner membrane. Extensive primary sequence mutagenesis of the OspA N-terminus revealed that, in

contrast to *E. coli*, the lipoproteins produced in *Borrelia* are localized to the surface of the cell by a default mechanism.

Analysis of the lipoprotein N-terminus revealed that it has a propensity for disorder, rich in residues that are not conducive to the formation of protein secondary structure. We term this region the lipoprotein 'tether' and show that removal of individual residues from within the OspA tether does not prevent transport of the lipoprotein across the periplasmic space but is detrimental to translocation of the lipoprotein across the outer membrane. We show that in addition to the surface, *B. burgdorferi* lipoproteins are also native to the periplasmic leaflets of the inner and outer membranes. This demonstrates the existence of an accurate system for localization of lipoproteins within the *Borrelia* cell envelope and we propose a two-step model for their transport to the cell surface. The work performed herein will serve as the basis for further analysis into the process of protein transport and pathogenesis for an important human pathogen.

Chapter I. Introduction

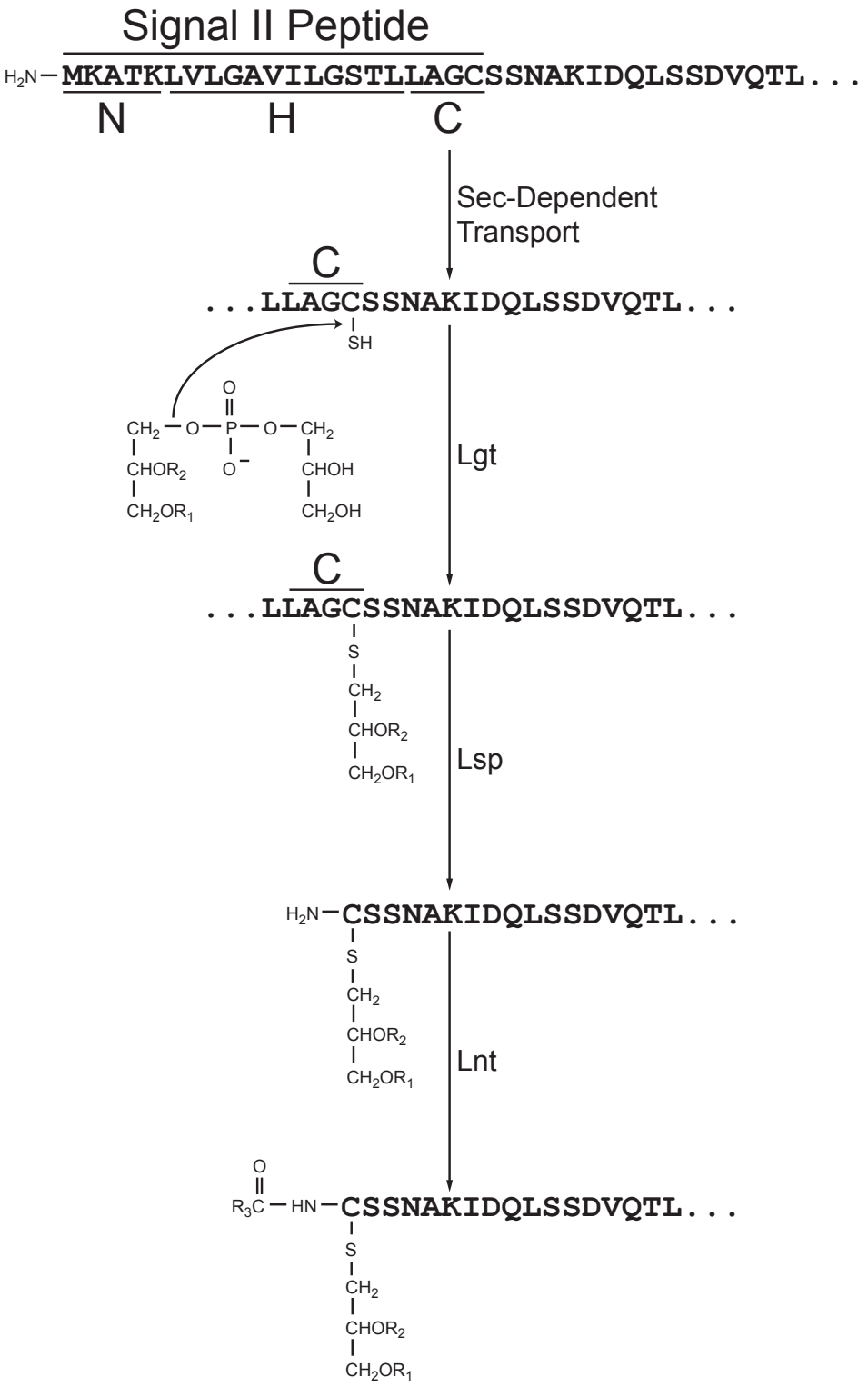
Stemming from the 1969 discovery and first description of murein lipoprotein by Volkmar Braun and colleagues, bacterial lipoproteins are now known to be numerous and shown to have wide variety of important functions (Braun,1969). Synthesized in the cytoplasm, all lipoproteins contain a cleavable signal sequence that directs them through the cytoplasmic membrane (Pugsley,1993). At this point, the covalent addition of a lipid tail provides the protein with membrane-associable characteristics that are ideal for cellular compartmentalization. Identified in all bacterial genomes to date, some organisms are estimated to encode for over 100 lipoproteins by themselves, some with important physiological roles but many of purely hypothetical function (Fraser,1997;Tjalsma,1999). In some bacteria, such as the Lyme disease spirochete *Borrelia burgdorferi*, lipoproteins are doubly important due to their roles in the establishment of infection (Brandt,1990;Probert,1998;Pal,2000;Pal,2004;Kraiczy,2004;Ramamoorthi,2005; Revel,2005). In bacteria with more than one membrane (i.e. Gram-negatives and others such as the *Borrelia spp.*), it is critical that a mechanism exists for proper localization of lipoproteins within the cell envelope. Much work on lipoprotein sorting has focused on transport within the periplasm of *Escherichia coli*. Almost nothing is known regarding the exposure of lipoproteins on the surface of *Borrelia burgdorferi*. The work contained herein represents an effort to understand this important process in a pathogen of increasing relevance.

Lipoprotein Biogenesis

Lipoproteins, like all other bacterial proteins, are first synthesized in the cytoplasm. A signal sequence (signal II peptide) is located at their amino-terminal end that specifically directs the prolipoprotein to the Sec translocon embedded in the cytoplasmic membrane (CM) (von Heijne,1989). The signal II peptide is characterized by the typical tripartite componentry of an amino-terminal positively-charged (N) domain, hydrophobic (H) core, and cleavage (C) region. Only the C-region differs significantly between lipoproteins and non-lipoproteins. In lipoproteins, the C-region is apolar and contains a consensus sequence of $L_{-3(}A/S)_{-2}(G/A_{)-1}\downarrow C_{+1}$ (von Heijne,1989). Though more recent studies have demonstrated degeneracy for the residues within the -3 to -1 positions, the cysteine residue following the cleavage site (Cys_{+1}) is invariant and absolutely necessary for the proper maturation of a lipoprotein (Nakai,1999;Falquet,2002;Sutcliffe,2002;Juncker,2003).

Following recognition by and passage of the prolipoprotein through the Sec apparatus, it undergoes a series of post-translational modifications that vary by bacterium but ultimately result in an N-terminally lipidated end product. In Gram-negative bacteria, a trio of CM-bound enzymes catalyze the formation of the mature lipoprotein (Fig. 1). Lgt (**l**ipoprotein diacyl**g**lyceryl **t**ransferase) transfers an *sn*-1,2-diacylglycerl group from phosphatidylglycerol (PG) to the sulfhydryl group of the cysteine at position +1 via a thioether linkage (Chattopadhyay,1977;Tokunaga,1982;Gan,1993;Sankaran,1994). **L**ipoprotein **s**ignal

Figure 1. Diagram of lipoprotein biogenesis in a typical Gram-negative organism. This figure shows the traditional steps taken in the maturation process of a lipoprotein. The positively-charged N region, hydrophobic core (H), and lipobox cleavage site (C) are labeled. Refer to text for details.


Signal II Peptide
H2N—MKATKLVLGAVILGSTLLAGCSSNAKIDQLSSDVQTL...
N
H
C
Sec-Dependent
Transport
C
...LLAGCSSNAKIDQLSSDVQTL...
SH
Lgt
C
...LLAGCSSNAKIDQLSSDVQTL...
Lsp
H2N—CSSNAKIDQLSSDVQTL...
Lnt
R3C—HN—CSSNAKIDQLSSDVQTL...

peptidase (Lsp) specifically recognizes the glyceride-modified prolipoprotein and subsequently cleaves the signal peptide such that the amino-terminus of the newly-formed apolipoprotein is a diglycerylcysteine residue (Tokunaga,1982;Yamada,1984). **L**ipoprotein ***N*-*acyl* t**ransferase (Lnt) performs the final step by removing an *N*-acyl group from membrane phospholipids (typically PG, but occasionally phosphatidylethanolamine (PE) and cardiolipin (CL)) and subsequently transferring it to the free amino group on the apolipoprotein's N-terminal diglycerylcysteine (Gupta,1991;Vidal-Ingigliardi,2007). The end result of these modifications is a triacylated protein localized to the outer leaflet of the cytoplasmic membrane.

Variations in this three-step process exist, especially amongst Gram-positive organisms. Although biochemical analyses have shown lipoproteins of *Bacillus subtilis* and *Staphylococcus aureus* to be *N*-acylated, homologues of the *lnt* gene are conspicuously absent (Hayashi,1985;Navarre,1996;Tjalsma,1999). Conversely, *Mycobacterium tuberculosis*, *Streptomyces coelicolor*, and *Corynebacterium glutamicum* all seem to have copies of *lnt*, yet *N*-acylation of lipoproteins in these organisms has yet to be conclusively demonstrated (Vidal-Ingigliardi,2007). The mycobacterial Lnt occurs together with a polyprenol monophosphomannose synthase (PPM synthase) as part of a two-domain protein (Rv2051c) (Gurcha,2002). It has been suggested that the Lnt domain in Rv2051c may have a more important role in the bioysnthesis of lipoarabinomannan and lipomannan, two key components of the

unique mycobacterial cell wall, than in lipoprotein biogenesis (Rezwan,2007;Hutchings,2008). An attempt at the complementation of an *E. coli* conditional *lnt* mutant with homologues found in the Gram-positive bacteria *S. coelicolor* and *C. glutamicum* was unsuccessful. The use of *lnt* from the Gram-negative bacteria *Yersinia tuberculosis*, *Neisseria meningitidis*, *Vibrio cholerae*, and *Pseudomonas aeruginosa*, however, did allow for growth of the *E. coli* mutant (Vidal-Ingigliardi,2007). These results suggest that *N*-acylation is more conserved and may play a more important role in lipoproteins of organisms containing more than one cell membrane. Supporting this idea are the findings that many Gram-positive organisms contain only diacylated lipoproteins and that they are able to tolerate knockouts of genes encoding all three enzymes (*lgt*, *lsp*, and *lnt*) involved in lipoprotein biogenesis (Leskela,1999;Petit,2001;Reglier-Poupet,2003; Baumgartner,2007). This is quite the opposite from Gram-negative organisms, for which each of the three appear to be universally essential (Yamagata,1982;Gan,1993;Gupta,1993;Sankaran,1994;Robichon,2005).

Differences in biosynthetic pathways extend prior to the lipid modification as some lipoproteins appear to use variations on traditional Sec-mediated export. The dimethyl sulfoxide reductase lipoprotein (DmsA) of the Gram-negative *Shewanella oneidensis* contains a **t**win-**a**rginine **t**ransport (Tat) signal within its N-terminus and has been shown to pass through the inner membrane of this organism together with its partner DmsB (Gralnick,2006). DmsA is therefore transported in a completely folded Sec-

independent fashion through the membrane (Lee,2006). Other lipoproteins have been demonstrated to use a less common SecA2-mediated mechanism of cytoplasmic membrane export. Very few proteins appear to require SecA2 versus the traditional SecA motor protein for transport across the CM. It is speculated that SecA2 may have a specialized role in the transport of factors related to Gram-positive pathogenesis (Lenz,2002;Braunstein,2003). Putative sugar uptake lipoproteins Msmeg1704 and Msmeg1712 of *Mycobacterium smegmatis* and two lipoproteins of unknown function in *Listeria monocytogenes* all appear to be dependent on the non-essential SecA2 for proper CM transport (Lenz,2003;Gibbons,2007).

Diverse functions for bacterial lipoproteins

The fatty acylation characteristic of all bacterial lipoproteins allows for sequestration in a membrane compartment. This is advantageous for the bacterium in that it can produce a protein and then tightly anchor it to its surface such that it isn't lost into the extracellular milieu. These surface-localized proteins can have functions related to adhesion, enzymatic activity, and immune evasion. Alternatively, if the protein is required as part of a multi-subunit complex, lipoproteins can shuttle components between membranes or act as key stabilization proteins anchored in the membrane to assist in the coordinated construction of large periplasm-spanning transport machinery.

Lipoproteins have important structural functions in bacteria. The well-characterized Braun's lipoprotein (Lpp) and **p**eptidoglycan-**a**ssociated **l**ipoprotein (Pal) are critical to maintenance of membrane integrity in *Escherichia coli*. The absence of either of these two proteins results in the sloughing off of membrane vesicles, leakage of periplasmic contents, and increased drug sensitivity due to improper linkage of the cell wall to the OM (Sonntag,1978;Lazzaroni,1981;Lazzaroni,1992;Bernadac,1998;Cascales,2002). MxiM is an OM pilotin lipoprotein of *Shigella flexneri* that aids in the assembly of the Type III Secretion (T3SS) needle apparatus. It functions to ensure the proper localization of MxiD subunits that oligomerize to form a ring-like OM secretin complex. True to its name, MxiM 'pilots' MxiD across the periplasm for specific insertion into the OM (Schuch,1999). Assembly of the MxiD secretin allows for downstream construction and secretion of important components through the T3S injectisome (Lario,2005;Okon,2008). A similar function has been attributed to the *Klebsiella oxytoca* pilotin PulS. In the absence of this lipoprotein, the PulD secretin is mislocalized in the inner membrane (Guilvout,2006). Tgl is a lipoprotein known to assist in the assembly of the *Myxococcus xanthus* secretin PilQ. Aside from PilQ assembly, Tgl may be unique in that it appears to also play a role in cell-cell communication through contact-dependent stimulation of secretin assembly (Nudleman,2005;Nudleman,2006).

The lipoproteins YfgL, NlpB, YfiO, and SmpA (BamB-E, respectively) of *E. coli* help stablize the structure of an important OM biogenesis complex, the YaeT/Omp85/BamA (β-**b**arrel **a**ssembly **m**achinery) complex (Voulhoux,2003;Wu,2005;Bos,2006;Malinverni,2006;Sklar,2007. The Bam complex is critical to the correct insertion of OM-spanning proteins, many of which are nutrient transporters of the bacterium. The importance of this process is demonstrated by the essentiality of YfiO/BamC and the fact that null mutants of the other three lipoproteins result in severe OM biogenesis defects due to permeabilization of the membrane (Wu,2005;Malinverni,2006;Sklar et al., 2007, Proc Natl Acad Sci U S A, 104, 6400-5;Vuong,2008).

Lipoproteins also play key roles in the virulence of certain pathogens. SphB1 is a surface-exposed lipoprotein of the whooping cough agent *Bordetella pertussis*. It is required for the enzymatic cleavage of filamentous hemagluttinin (FhaB), the major antigen of *B. pertussis*, into its mature form (Coutte,2001;Coutte,2003;Mazar,2006). Both MtrC and OmcA assist in the reduction of solid metal oxides in and are found on the surface of the bacterium *Shewanella oneidensis* (Shi,2008).NalP of *Neisseria meningitidis* is a proteolytic surface lipoprotein required for the proper maturation of two proteins, App (an adhesin expressed during human colonization) and IgA protease (van Ulsen,2001;van Ulsen,2003). SphB1, MtrC, OmcA, and NalP are rare amongst bacterial lipoproteins by virtue of their surface localization. The presence of lipoproteins on the cell exterior is not unusual for Gram-positive organisms due to

their single membrane. Most bacterial lipoproteins of Gram-negative organisms, however, are found within the confines of the periplasmic space. In addition to these four examples, the majority of lipoproteins from another pathogen, *Borrelia burgdorferi*, are also found on its cell surface.

Borrelia burgdorferi and Lyme disease

Borrelia burgdorferi is the pathogen responsible for Lyme disease in North America. Lyme disease is a nationally notifiable disease in the United States based on rising numbers of infections across North America and Europe. In the 15-year period between 1992 and 2006, nearly 250,000 cases of Lyme disease were reported to the Centers for Disease Control (CDC) (Bacon,2008). The classification of Lyme disease as an "emerging infectious disease" is perhaps misleading when one considers that symptoms arising from infection with borreliae have been detailed for quite some time and that physicians have been wrangling for the better part of 130 years to pinpoint the cause of its infectious sequelae. In 1883, the German physician Alfred Buchwald was the first to describe the symptoms of infection as a "diffuse idiopathic skin atrophy" (Buchwald,1883). This condition, with symptoms causing the skin to take on a thin, wrinkled "tissue-paper-like" appearance is now well understood to be a late-stage manifestation of European Lyme borreliosis (Steere,2001). In late October of 1909, at a meeting of the Swedish Dermatological Society, Arvid Afzelius presented data on a curious rash that tended to spread outward on the skin into a bullseye-like pattern. He referred to this condition as "erythema migrans (EM)," the

first usage of a term that is characteristic of and diagnostic for *B. burgdorferi* infections to this day (Afzelius,1910;Tibbles,2007). Afzelius later hypothesized that the rash might in some way be connected to the bite of a tick (Afzelius,1921). Meanwhile, in North America, Wisconsin physician Rudolph Scrimenti, who had been following Europe's cases and reports of its rash of unknown origin, was presented with a case of EM and noted for the first time that he was able to successfully treat the patient with a regimen of penicillin antibiotics (Scrimenti,1970). This was the first clear indication that EM might be related to a bacterial infection and was also the first documented case of EM in North America. It was not until seven years later that an outbreak of juvenile rheumatoid arthritis struck the community of Old Lyme, Connecticut. Careful analysis of those cases led to the discovery of a previously unknown spirochete and the conclusive link five years later between the pathogen (*B. burgdorferi*) and so-called Lyme disease (Steere,1977;Burgdorfer,1982).

The clinical presentation of Lyme disease is varied, making early diagnosis somewhat difficult. The single clear diagnostic sign of infection is the tell-tale EM/bulls-eye rash, though oftentimes it will go unnoticed by the patient (if it even appears in the first place – approximately twenty percent of infections do not result in the EM rash (Steere,2001)). Accompanying the rash may be numerous non-specific symptoms including, but not limited to: depression, headache, muscle pain, generalized malaise, fatigue, and arthralgia (Steere,1989;Steere,2001). If left untreated, the disease can

take a variety of courses. Involvement in the skin is very common, but there are key differences in disease progression for North American and European cases of infection. This is widely attributed to the fact that different genospecies of *Borrelia* are implicated on the two continents: *B. afzelii*, *B. garinii*, and *B. burgdorferi* in Europe and *B. burgdorferi* alone in North America (Steere,2001). Skin lesions are typically limited to a rapidly spreading EM rash in North America, whereas European Lyme borreliosis can additionally result in lymphocytoma or acrodermatitis chronica atrophicans (Nadelman,1998). Other manifestations of Lyme disease arise in the heart (Lyme carditis), the nervous system (neuroborreliosis), and frequently in the joints (Lyme arthritis) (Sigal,1995;Logigian,1990;Steere,2001). For the great majority of patients, Lyme borreliosis is a non-fatal but debilitating disease. Nearly ten percent of patients with Lyme arthritis report pain for a duration of greater than one year (Rees,1994). Diagnosed early enough, it is easily and consistently treatable with antibiotic therapy (Seltzer,2000;Steere,2001). Only a vanishingly small number of patients, typically those who experience an atrioventricular heart block due to Lyme carditis, die as a result of borrelial infection (Steere,1989).

***Physiology of* B. burgdorferi**

Borrelia burgdorferi is a member of the order Spirochetales and belongs to the same family (Spirochetaceae) as *Treponema pallidum*, the agent of syphilis. *Leptospira interrogans*, the cause of leptospirosis, is a close relative from another family, the Leptospiraceae. Prior to the discovery of *B. burgdorferi*, spirochetes had been studied

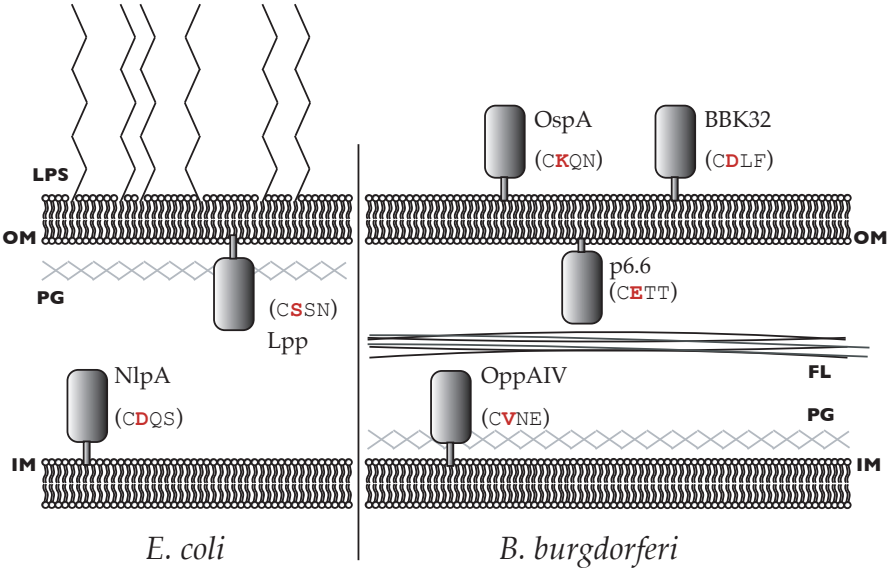
for some time, dating back to the original description of the genus *Spirochaeta* by Christian Gottfried Ehrenberg in 1835 (Ehrenberg,1835). Syphilis, a disease known in literature to date from at least the fifteenth century, was not conclusively linked to *T. pallidum* until the early twentieth century (Noguchi,1912). The spirochetes are therefore both historically and evolutionarily an ancient phylum of the bacterial kingdom. This phylogenetic antiquity is manifest in a bacterial morphology and membrane architecture that are strikingly different from traditional Gram-positive and Gram-negative organisms.

The dimensions of *B. burgdorferi* vary between 100nm and 300nm in width by 10μm and 25μm in length (Holt,1978;Goldstein,1996). This is dramatically different from bacteria such as the prototypical 0.5-1μm x 2μm rod-shaped *E. coli*: the narrow width of *Borrelia* falls below the wavelength of visible light and therefore precludes direct visualization of the organism by light microscopy. When viewed under phase contrast or darkfield microscopy, the flat waveform morphology unique to this phylum is clearly visible. The corkscrew-like shape greatly enhances the ability of spirochetes to traverse viscous media and burrow deep into tissues such as the connective tissue found within joints (Berg,1979;Goldstein,1994;Motaleb,2000;Nakamura,2006). The spiral shape is due in part to the presence of endoflagella anchored to each pole of the bacterium (axial filaments) (Holt,1978;Charon,2009). These unique flagella are entirely enclosed within the periplasmic space of the spirochete and have terminal motors operating in opposing directions to provide motility

(Charon,2002;Charon,2009). So important to spirochetal survival are its flagella that an estimated six percent (54 ORFs) of the *B. burgdorferi* genome is dedicated to genes involved in motility (Fraser,1997).Yet they are not the only unique characteristic of the *Borrelia* ultrastructure. Unlike Gram-negative organisms, which contain a thin peptidoglycan layer in close association with the OM, the cell wall of *Borrelia* seems to instead be more tightly associated with the IM (Holt,1978). Also unlike the OM of Gram-negatives is the complete absence of lipopolysaccharide from the outer leaflet (Takayama,1987). See Fig. 2 for an illustration of these unique characteristics.

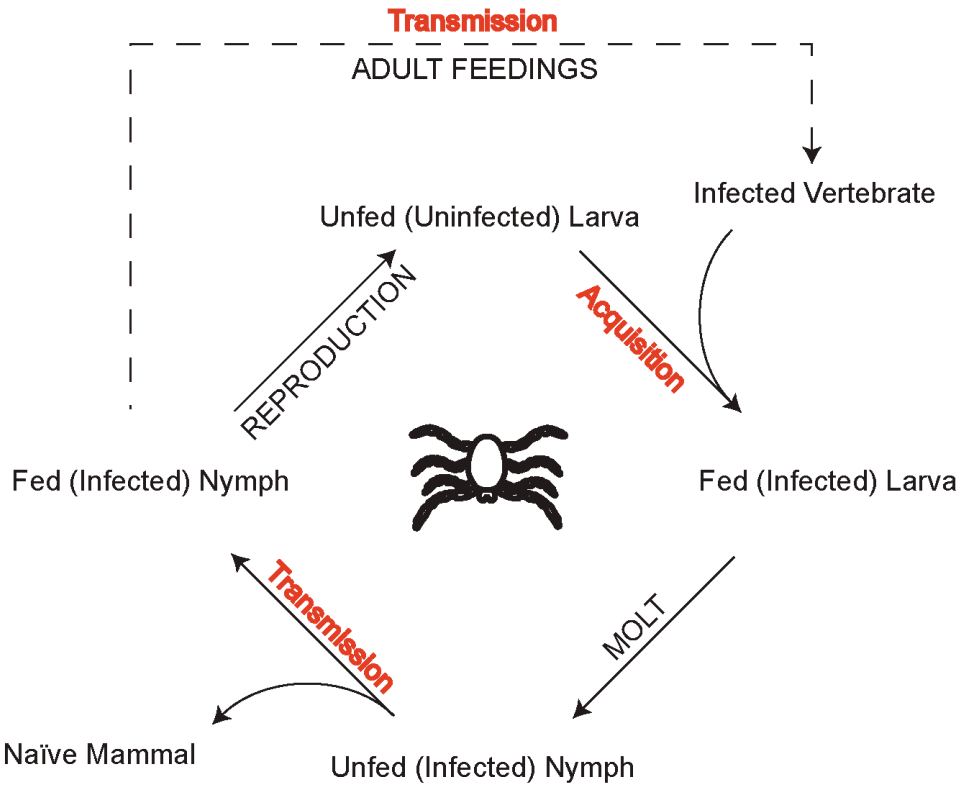
In addition to its membrane architecture, *B. burgdorferi* also has a genetic organization that is arguably one of the most complex in bacteria, even when compared to other members of its own family. The genome itself is highly segmented with a linear chromosome of less than 1Mb and numerous extrachromosomal circular and linear plasmids of varying sizes (Fraser,1997;Casjens,2000;Rosa,2005). With one exception, the >20 plasmids are dispensable for *in vitro* bacterial growth, though contributions of each to the relative pathogenicity of a given *B. burgdorferi* strain have been identified (Purser,2000;Labandeira-Rey,2001;Rosa,2005). A 26-kb circular plasmid (cp26) contains the essential *resT* telomere resolvase that aids in resolution of the hairpin ends of the linear chromosome and is present in all known strains of *B. burgdorferi* (Kobryn,2002;Byram,2004;Jewett,2007). The genome itself

Figure 2. Comparison of *Escherichia coli* and *Borrelia burgdorferi* membrane architecture. This is a simplified representation of some of the major differences between the membrane organizations for the two organisms. Labels refer to: outer membrane (OM), inner membrane (IM), peptidoglycan (PG), flagella (FL), and lipopolysaccharide (LPS). Representative lipoproteins from each organism are shown, along with the first four residues of each mature sequence. Highlighted in red are the +2 position amino acids, known to be important for sorting in *E. coli* but not in *Borrelia.*


LPS
OM
PG
(CSSN)
Lpp
NlpA
(CDQS)
IM
OspA
(CKQN)
BBK32
(CDLF)
OM
p6.6
(CETT)
FL
OppAIV
(CVNE)
PG
IM
E. coli
B. burgdorferi

is very A/T rich, with a G/C content of only 28.6% (Fraser,1997). Though not large in size, the genome does encode for a proportionally high number of lipoproteins, with nearly 10% dedicated to known or hypothetical lipoproteins (Fraser,1997). Many of these lipoproteins have no known homologues and it is thought that they may contribute to spirochete-specific mechanisms of pathogenicity. *B. burgdorferi* exists within an enzootic cycle together with its vectors, species of *Ixodes* ticks. This life cycle has now been well-characterized and shown to be heavily dependent on the correct trafficking of key lipoproteins to the bacterial cell surface. The bacterium is intimately associated together with its arthropod vector during a series of moltings and feedings. The lifespan of a typical *Ixodes spp.* tick is approximately two years and consists of three distinct stages: larval, nymphal, and adult (Fish,1995;LoGiudice,2003). Typically, entry of *B. burgdorferi* into the zoonotic cycle occurs upon the feeding of an infected mammalian reservoir (often the white-footed mouse, *Peromyscus leucopus*) by the deer tick, *Ixodes scapularis* (Barbour,1993;LoGiudice,2003). The feeding process takes approximately four days and during this time, the bacterium is ingested by the tick (within 48 hours of feeding start) and establishes residency in the arthropod's midgut (Piesman,1987). After completion of a bloodmeal, the tick will undergo the process of molting and move on to the nymphal stage. It is at this point that the tick, harboring the spirochete in its digestive tract, is at its most aggressive in feeding and consequently this is the stage at which transmission to humans frequently occurs (Wormser,2006). The nymphal tick will spend approximately one year in an adult stage, with the ability to transmit the

Figure 3. Enzootic lifecycle of *Borrelia burgdorferi* and *Ixodes spp.* ticks. A simplification of the vector-host cycle in which *B. burgdorferi* takes part. The entire cycle of tick development (from egg to larva to nymph to adult) takes approximately two years. The tick is at its most aggressive during the unfed nymphal state; consequently this when most accidental transmissions of the spirochete to humans occur.


Transmission
ADULT FEEDINGS
Infected Vertebrate
Unfed (Uninfected) Larva
REPRODUCTION
Acquisition
Fed (Infected) Nymph
Fed (Infected) Larva
Transmission
MOLT
Naïve Mammal
Unfed (Infected) Nymph

disease upon each feeding. Reproduction leads to the female tick laying eggs and starting the cycle once again. There is no documented evidence of transovarial transmission as occurs in *Ricketsia rickettsii*, therefore the larval ticks must reacquire *B. burgdorferi* from a competent reservoir and thus the process continues (Schwan,2000).Because of the extreme environmental changes encountered as it passes from vertebrate reservoir to arthropod vector and back, there is a necessity for a mechanism to allow the bacterium to shield itself from clearance in each situation. Recent studies have clarified the means by which *B. burgdorferi* is able to accomplish this and the underlying solution appears to be centered around the presence of highly specialized surface lipoproteins, especially OspA and OspC.

OspA

The *ospA* gene is present on a 54-kilobase linear plasmid (lp54) and encodes for a polypeptide containing a signal-II peptidase cleavage site, required for lipidation of the cysteine residue at position 17 (lipo-Cys17) (Howe,1985;Bergstrom,1989;Brandt,1990). The mature form of OspA (processed and lipidated) is a 28kDa (257 amino acid) lipoprotein with significant antiparallel β-sheet structure and a single carboxy-terminal α-helix (Li,1997). The amino-terminal twelve residues do not appear in the crystal structure on the basis of their weak electron density and are presumed to be disordered (Li,1997). At the C-terminal end are two residues, Phe237 and Ile243 which are brought into close apposition upon folding of the OspA polypeptide (Pal,2000). These two residues are part of a

hydrophobic pocket that binds to a receptor found within the tick midgut. This receptor, TROSPA (**t**ick **r**eceptor for **OspA**), is required for colonization of *I. scapularis* and undergoes a cycle of expression which closely mirrors that of spirochete presence in the tick's digestive system (Pal,2000;Schwan,2000;Pal,2004). Upon infection of the tick, mRNA levels of TROSPA are seen to increase within the tick and are seen to decrease upon ingestion of the next bloodmeal, presumably facilitating the exit of OspA-expressing *B. burgdorferi* from the vector's midgut (Pal,2004). The signal for decreasing TROSPA mRNA production is thought to be the temperature shift encountered upon feeding (a shift from approximately 34°C to 37°C) (Pal,2004). This same temperature has also been shown by *in vitro* studies to cause *B. burgdorferi* to switch the dominant antigen on its cell surface from OspA to OspC (Schwan,2000).

OspC

OspC is a variably expressed 23kDa protein that has been shown to be important in the early phases of mammalian infection. Like OspA, the gene encoding this protein is located on an extrachromosmal plasmid. *ospC* is found, interestingly enough, on a 26kb circular plasmid (cp26) that is stably maintained in all strains of *B. burgdorferi* (Schwan,2000;Pal,2004). This would seem to indicate that an extremely important function might be ascribed to the gene product. Despite the fact that OspC is one of the most highly expressed proteins in *B. burgdorferi*, its function is not as clearly understood as OspA. Following ingestion of a bloodmeal, *B. burgdorferi* undergoes a

rapid reorganization of its lipoprotein repertoire and exchanges the OspA, which previously coated the cell surface, with OspC. Accompanying this change, the spirochete detaches from its receptor in the tick midgut and migrates to the salivary glands (Pal,2004;Grimm,2004;Liang,2004). A study using an *ospC* knockout strain of *B. burgdorferi* showed that this mutant was unable to bind to *I. scapularis* salivary gland tissue. Complementation of the missing gene with a copy introduced on an extrachromosomal plasmid restored binding to the salivary glands at levels equivalent to the wild-type strain (Pal, 2004). A protein produced by the tick, Salp15, is known to inhibit T-cell inactivation and was recently demonstrated to bind tightly to OspC while the spirochete migrated through the tick's salivary glands. This finding may shed light on the role for OspC in promoting early stages of mammalian infection, as borreliae migrating from the tick may be shrouded in Salp15 (Ramamoorthi,2005;Hovius,2008).

Once transmission of the spirochete to the host is complete, later requirements for OspC remain poorly understood. One study showed that complementation of an *ospC* knockout strain with an unstable copy of *ospC* had no effect on the ability of the spirochete to infect naïve mice. Spirochetes recovered from the infected mice did not contain the plasmid-encoded *ospC*. This result suggests that OspC is only critical for very early stages of mammalian acclimatization and that the presence of this protein at later stages is unnecessary for infection to proceed (Tilly,2006). Another study used an *ospC* knockout strain grown inside an immunologically privileged peritoneal

dialysis chamber that had been implanted into rats. The spirochetes showed no difference from wild-type or complemented strains in their ability to grow and adapt inside the chamber (Stewart,2006). Though this shows that OspC is not required for spirochete survival inside the mammalian host, it does not clarify whether a physiological function for the protein exists, such as complement evasion (Stewart, 2006). Intradermal injection of *ospC* $^{-}$ spirochetes into C3H-HeN mice were cleared rapidly (within 24h) whereas wild-type *B. burgdorferi* was isolated at all subsequent time points and from distal locations, indicating that OspC does seem to play a role in the early stages of mammalian infection (Tilly,2007). It is likely that this lipoprotein plays no role in protection from the adaptive immune response as it is typically not expressed by the bacterium for a period of time that is long enough to be targeted by antibodies. In fact, constitutive expression of *ospC* has been shown to be detrimental to spirochete survival in immunocompetent mammals (Xu,2006).

Protein trafficking in bacteria

The presence of OspA and OspC on the surface of *B. burgdorferi* illustrates a solution to a classic problem faced by bacteria, namely how to transport a protein produced in the cytoplasm through multiple lipid bilayers to the cell surface or beyond. Numerous mechanisms have been devised by bacteria for just this purpose, occasionally encoding complex systems dedicated in some cases to the transport of only a single known protein (Odenbreit,2000;Driessen,2001;Pugsley,2004;Economou,2006). Pathogens are especially well-equipped to selectively secrete key proteins into their

surroundings that promote their survival in different ways. Toxins, adhesins, and proteases are just a few of the various types of secreted proteins seen in bacteria, and many of them are transported with the help of dedicated mechanisms that have evolved to allow for transport to occur precisely when required by the bacterium for a given condition (Galan,1999;Durand,2005;Mougous,2007). There are thought to exist several so-called "secretion systems" that are all unique in their compositions and mechanisms of action but similar in that they all function to move a given protein from the cytoplasm to the surface of the cell or beyond. At least seven different types of secretion systems (**T**ype **1** to **T**ype **7** **S**ecretion **S**ystem, T1SS → T7SS) have been described in the literature (For reviews on these systems, see Henderson,2004; Christie,2005;Holland,2005;Economou,2006;Johnson,2006;Mougous,2006;Putkatzki, 2006;Cornelis,2006;Abdallah,2007).

Of the seven types of secretion systems, there are three (T2SS, T4SS, and T5SS) that are wholly (T2SS, T5SS) or partly (T4SS) dependent on the proper function of the Sec/Tat translocation machinery for the early steps in the movement of a given protein to the cell exterior (Economou,2006). The primary role for the Sec/Tat systems is to move a polypeptide across the cytoplasmic membrane (Driessen,2001;Ize,2002;Pugsley,2004). For Gram-positive organisms, this is equivalent to moving a protein to the cell surface. For Gram-negative bacteria, the proteins enter the periplasmic space. The Sec system is the predominant mechanism by which proteins are moved across the cytoplasmic membrane in both Gram-positive

and Gram-negative organisms. The Sec machinery consists of a membrane-bound SecYEG complex which functions as a pore through which polypeptides are funneled (van den Berg,2004;Luirink,2005;Papanikou,2007). ATP hydrolysis provided by the SecA motor protein provides the energy necessary to ratchet the unfolded protein through the membrane (Zimmer,2008;Erlandson et al., 2008, J Biol Chem, 283, 15709-15;Erlandson et al., 2008, Nature, 455, 984-7). The nascent protein can then either be inserted laterally into the cytoplasmic membrane by the actions of YidC or is otherwise released into the periplasm for further transport or function (Luirink,2005;Xie,2008). Another mechanism, the Tat system, relies on the presence of a special twin-arginine motif present in the N-terminal signal peptide that directs the protein to a special complex designed for the transport of folded proteins across the CM (Thomas,2001;Ize,2002;Lee,2006;Tullman-Ercek,2007). Other systems (T1SS, T3SS, T4SS, T6SS, T7SS) are organized in such a fashion that transport of the protein across the cytoplasmic membrane bypasses the Sec machinery altogether. Foremost among these mechanisms is the well-characterized Type 3 Secretion System (T3SS). Used by pathogens such as *Yersinia pestis* (plague), *Salmonella typhimurium* (gastroenteritis), *Shigella flexneri* (bacillary dysentery), and *Bordetella pertussis* (whooping cough), the characteristic needle complex (injectisome) has the ability to directly transfer effector proteins into the host cell cytosol (Cornelis,2006).

Outer-membrane specific proteins that are to be inserted into the membrane, but not secreted are carried across the periplasm of Gram-negative bacteria via the Skp and

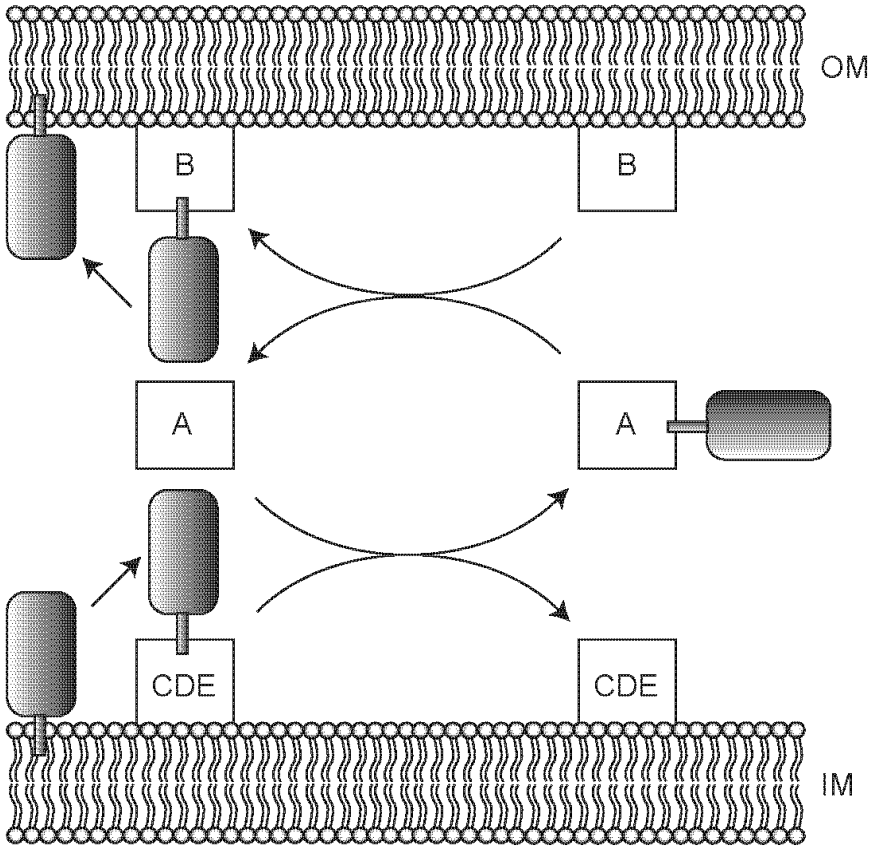
SurA chaperones (Bos,2006). Skp and Sur aid in the process of taking OM proteins from the Sec system and directing them to the OM protein assembly complex known as the Bam complex (B-barrel assembly machinery) (Sklar et al., 2007, Genes Dev, 21, 2473-84). Interaction of the C-termini of OMPs with the N-terminal POTRA domains of the BamA protein result in the folding and membrane insertion of the OMP (Voulhoux,2003;Bos,2006;Robert,2006;Kim,2007;Gatzeva-Topalova,2008).

Lipoprotein transport

Many Gram-negative lipoproteins have no transmembrane domains and are sorted to and inserted into the OM by a different process. Lipoproteins appear to use a combination of the Sec machinery and a dedicated transport system, the Lol system for lipoprotein localization (Narita,2004;Tokuda,2004). The Lol pathway was originally worked out experimentally in *E. coli* but has since been shown to likely be a near-universal system for proper localization of lipoproteins in organisms with more than one membrane (diderms). The prototypical Lol machinery is made up of five components, an ABC-like transporter, LolCDE, a periplasmic chaperone, LolA, and an OM receptor, LolB (Matsuyama,1995;Matsuyama,1997;Yakushi,2000).

After the lipoprotein exits the Sec translocon and is modified by the action of Lgt, Lsp, and Lnt, the process of localization occurs. The sorting of a lipoprotein in *E. coli* and other species of the family Enterobacteriaceae seems to be heavily influenced by

Figure 4. Lol-system-mediated lipoprotein transport to the *E. coli* outer membrane. Transport of a lipoprotein from the cytoplasmic membrane to the inner leaflet of the outer membrane requires a five-component system named Lol. A lipoprotein bound for the OM is first recognized by an ABC-transporter-like complex (LolCDE) in the IM. The lipoprotein is passed off to a periplasmic chaperone (LolA) in a 1:1 water-soluble complex. The affinity of the outer membrane receptor (LolB) for the acylated moiety of the lipoprotein is very high; it receives the lipoprotein and inserts it into the periplasmic face of the OM.


OM
B
B
A
A
CDE
CDE
IM

the identity of the residue immediately adjacent to the fatty-acylated N-terminal cysteine (Tokuda,2004;Lewenza,2006). An aspartic acid residue at this '+2' position is sufficient to cause lipoprotein retention in the cytoplasmic membrane (Yamaguchi,1988;Gennity,1991. Most other +2 residues support recognition by LolCDE and are sorted to the OM, where they are inserted by the OM receptor, LolB. The basis for Asp_{+2} retention seems to be a result of an inability to interact with the LolCDE complex and therefore may be a passive avoidance mechanism rather than active retention. It has been proposed that the negative charge of the residue is necessary but not sufficient for retention as a Glu_{+2} signal does not result in IM localization (Terada,2001). Hara *et al.* showed that the interatomic distance between the Cα and negative charge of the Asp side chain (2.72 – 3.69Å) is critical to IM localization of the lipoprotein (Hara,2003). In addition, a role for the positively-charged phosphatidylethanolamine (PE) head group was proposed on the basis of the observation that Asp_{+2} no longer functioned as a retention signal when PE had been treated with sulfo-*N*-hydroxysuccinimide acetate, an amine-specific reagent (Hara,2003).

Certainly Asp_{+2} may be a potent lipoprotein IM retention signal, but it has been clearly demonstrated that it is not the only residue that results in LolCDE avoidance. Using a lipidated variant of the *E. coli* maltose binding protein MalE, Seydel *et al.* convincingly showed that Phe, Pro, Trp, and Tyr were just as effective in retaining the reporter lipoprotein in the IM (Seydel,1999). Though these four residues might be

effective as retention signals, they are not found at the +2 position of any known *E. coli* lipoproteins. The strength of the avoidance signal has the potential to be weakened if Ala, Cys, His, Ile, Lys, or Thr are present at the +3 position. Conversely, when not present at the +2 position, Asp_{+3} appears to be equally, if not more effective provided the residue at position +2 is Asp, Glu, Gln, or Asn (Tokuda,2004). Ambiguity exists for other residues, such as Gly_{+2}, which seems to localize lipoproteins to both membranes regardless of whether Asp is at the second position(Seydel,1999). These findings suggest that there be the potential for fine-tuning of lipoprotein localization in *E. coli* and that the process of lipoprotein sorting may not be a strictly OM/IM binary decision.

Outside of the Enterobacteriaceae, very little is understood about the signals that govern lipoprotein sorting. Work in *Pseudomonas aeruginosa* suggests that the residues at the +3 and +4 positions may hold more importance to localization in that organism (Narita,2007). Other organisms seem to contain an incomplete set of the Lol proteins. *Neisseria gonorrhoeae*, *Bordetella pertussis*, and *Geobacter sulfurreducens* are apparently missing a copy of LolE. *Desulfovibrio vulgaris* and *Brucella suis* are additionally missing a copy of the OM LolB receptor (Narita,2006). Homologues for LolA and LolCDE have been identified in *Borrelia burgdorferi* but no LolB homologue has been found. As no information currently exists for the process of sorting lipoproteins in *B. burgdorferi*, the work presented here represents the first true

steps in identifying the basis for localization of the important lipoprotein virulence factors for this organism.

The experiments performed in this body of work are geared to the identification of the basic underlying tenets of lipoprotein sorting in *B. burgdorferi*. Chapter II presents first insights into the sequence-specific rules governing membrane localization of lipoproteins and reveals that *B. burgdorferi* may possess a default mechanism for lipoprotein transport to its cell surface. We introduce the possibility that inner-membrane retention of lipoproteins may also be governed by the presence of negatively-charged residues, but only in an appropriate context. Chapter III expands on this knowledge by further analyzing the unstructured region at the N-terminus of bacterial lipoproteins and presenting evidence that it plays a role in regulation of OM translocation. This role, in contrast to regulation of IM release, may be sequence-independent in nature. Chapter IV examines the role of lipoproteins from subsurface compartments in *Borrelia* and introduces an assay that takes advantage of such lipoproteins to aid in the discovery of novel sorting signals. Chapter V uses epitope-tagged mutants generated in the research from Chapter III for the purposes of making a first attempt at identifying components of a putative OM lipoprotein translocase. This research therefore represents a progression from first understanding the principles used to determine whether a lipoprotein is sorted to determining the mechanism by which the proteins are physically sorted. We present a final model based on our findings that suggests the existence of a two-step recognition process for lipoprotein secretion to the cell surface. Together, the data gathered here will serve as

a basis for more detailed examinations of lipoprotein transport in this important pathogen.

The following chapter was published as

Schulze, R. J., and W. R. Zuckert. 2006. Borrelia burgdorferi lipoproteins are secreted to the outer surface by default. *Mol Microbiol* **59**:1473-1484.

Chapter II: *Borrelia burgdorferi* Lipoproteins are Secreted to the Outer Surface by Default

Abstract

Borrelia spirochaetes are unique among diderm bacteria in their abundance of surface-displayed lipoproteins, some of which play important roles in the pathogenesis of Lyme disease and relapsing fever. To identify the lipoprotein sorting signals in *Borrelia burgdorferi,* we generated chimeras between the outer surface lipoprotein OspA, the periplasmic oligopeptide-binding lipoprotein OppAIV and mRFP1, a monomeric red fluorescent reporter protein. Localization of OspA and OppAIV point mutants showed that *Borrelia* lipoproteins do not follow the "+2" sorting rule which targets lipoproteins to the cytoplasmic or outer membrane of gram negative bacteria via the Lol pathway. Fusions of mRFP1 to short N-terminal lipopeptides of OspA, and surprisingly OppAIV, were targeted to the spirochaetal surface. Mutagenesis of the OspA N-terminus defined less than five N-terminal amino acids as the minimal secretion-facilitating signal. With the exception of negative charges, which can act as partial subsurface retention signals in certain peptide contexts, lipoprotein secretion occurs independent of N-terminal sequence. Together, these data indicate that *Borrelia* lipoproteins are targeted to the bacterial surface by default, but can be retained in the periplasm by sequence-specific signals.

Introduction

Throughout their vector-host lifecycle, *Borrelia* spirochaetes causing tick-borne Lyme borreliosis and relapsing fever display abundant surface lipoproteins, which are anchored in the bacterial outer membrane lipid bilayer via an N-terminal triacyl-modified cysteine (Brandt,1990;Beermann,2000). Some of these lipoproteins are important virulence factors during transmission, colonization and persistence of these bacterial pathogens. For example, the outer surface protein A (OspA) of the Lyme borreliosis agent *Borrelia burgdorferi* (Howe,1985) is involved in bacterial colonization of the tick midgut (Pal et al., 2000; Pal et al., 2001; Pal et al., 2004) and has been used as a first-generation, transmission-blocking Lyme disease vaccine for humans (Steere et al., 1998). In contrast, OspC is upregulated during tick feeding and involved in establishing mammalian infection (Schwan and Piesman, 2000; Gilmore and Piesman, 2000;Pal et al., 2004;Grimm et al., 2004;Ramamoorthi et al., 2005). The variable large (Vlp) and small (Vsp) proteins of relapsing fever agents such as *Borrelia hermsii* or *Borrelia turicatae* are immunodominant and antigenically distinct lipoproteins, which allow the spirochaetes to repeatedly evade the host's immune reponse by multiphasic antigenic variation and to target different tissues (Cadavid et al., 2001; Barbour, 2003).

Despite the obvious importance of surface lipoproteins for *Borrelia* pathogenesis, the sequence of molecular events leading from their cytoplasmic expression to their emergence on the bacterial surface as biologically active molecules remains largely unknown. We recently showed that recombinant *B.*

burgdorferi can express and present on their surface biologically active relapsing fever *Borrelia* Vsps and Vlps (Zückert et al., 2004). This demonstrated that the lipoprotein transport machineries in the genus *Borrelia* are functionally conserved. Detailed studies on lipoprotein secretion in other diderm, i.e. double-membrane bacteria (Gupta, 1998) have been limited to a few model proteins, which are transported by either a type II or an autotransporter secretion pathway (Pugsley, 1993;Coutte et al., 2003;van Ulsen et al., 2003). Yet, homologs of a type II secretion apparatus, other Sec-dependent or –independent secretion pathways are absent from the *B. burgdorferi* genome (Fraser et al., 1997;Casjens et al., 2000;Haake, 2000), and known surface *Borrelia* lipoproteins do not contain C-terminal structural features of autotransporter translocator domains (Oomen et al., 2004).

Based on the identification of Lol homologs in *B. burgdorferi* (Masuda et al., 2002), we previously suggested that *Borrelia* spirochetes instead use a Lol-like system to transport lipoproteins (Zückert et al., 2004). The *Escherichia coli* Lol pathway sorts major lipoproteins such as Braun's lipoprotein Lpp (Braun and Rehn, 1969) to the periplasmic leaflet of the outer membrane (OM) via the Lol pathway (Tokuda and Matsuyama, 2004). Lipoprotein sorting within the periplasm is based on the properties of the amino acid following the N-terminal, acylated Cys, i.e. the so called '+2 rule', with Asp serving as the canonical inner membrane (IM) retention signal (Yamaguchi et al., 1988). Pro or aromatic amino acids Phe, Trp, Tyr can also act as retention signals (Seydel et al., 1999). On the other hand, Ser or other amino acids at the same position act as OM signals. Amino acids at position +3 can either

weaken (Lys) or strengthen (Asp, Glu, Gln, Asn, Arg) IM retention by avoiding interaction with the Lol system (Masuda et al., 2002; Terada et al., 2001). However, mislocalization of *B. burgdorferi* surface lipoproteins to the *E. coli* IM and the analysis of primary sequences of spirochaetal lipoproteins (Dunn et al., 1990; Haake, 2000) suggested that sorting signals are different from those found in other diderm, i.e. double-membrane bacteria (Gupta, 1998).

In this study, we used fusions of amino terminal lipopeptides to a monomeric red-fluorescent reporter protein to visualize lipoproteins in the periplasm and on the surface of living spirochaetes. This allowed us to determine readily the cellular localization of *B. burgdorferi* surface and sub-surface lipoprotein mutants, granting the first intriguing insights into the molecular mechanisms of spirochaetal lipoprotein targeting.

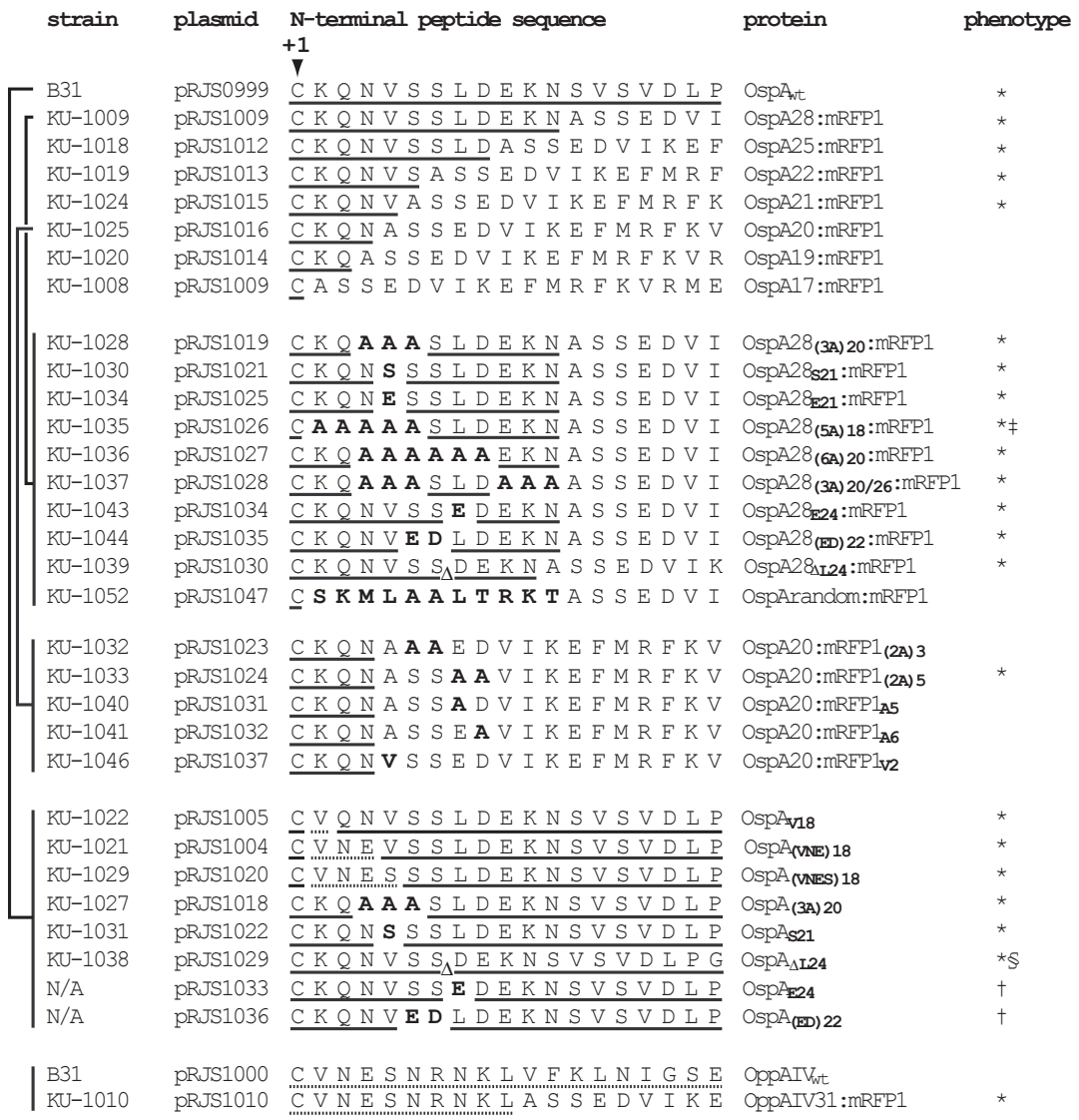
Results

***B. burgdorferi* Lipoprotein Sorting Does Not Follow the '+2 Rule'.** The linear chromosome and numerous linear and circular plasmids of *B. burgdorferi* harbour genes for more than 130 potential lipoproteins (Casjens et al., 2000; Fraser et al., 1997). Our sequence analysis of 31 known lipoproteins indicated that the sorting rules for *E. coli* lipoproteins cannot be applied to predict the actual localization of *B. burgdorferi* lipoproteins. While over 75% of these lipoproteins have "OM-permitting" Lys or Asn residues at position +2, at least two known surface-exposed lipoproteins, the fibronectin-binding BBK32 (Probert and Johnson, 1998) and P27

(Reindl et al., 1993) have an "IM-retaining" Asp at this position. A simple prediction of other amino acids serving as borrelial retention signals failed as well: the oligopeptide-binding lipoprotein OppAIV is localized to the periplasm (Bono et al., 1998) and has a Val at position +2, but so does OspD, a described outer surface protein (Norris *et al*., 1992). This led us to hypothesize that the sorting of borrelial lipoproteins is governed by different amino acid determinants, and we took an empirical and step-wise approach to identify these rules.

As model surface and subsurface lipoproteins, we chose two lipoproteins with well-documented cellular localization, OspA (Barbour et al., 1983) and OppAIV (Bono et al., 1998). We initially focused on OspA and OppAIV differences in positions +2 to +4 and swapped the OspA tripeptide $K^{18}Q^{19}N^{20}$ with the OppAIV sequence VNE in an attempt to mislocalize OspA (Figs 5 and 6). A mutant $ospA_{(VNE)18}$ gene was generated by sequence overlap extension (SOE) PCR of a pBSV2 P_{flaB}-*ospA* construct and transformed into *B. burgdorferi* B313, which lacks lp54 and therefore its endogenous *ospA* copy (Sadziene et al., 1995). Like wild type (w.t.) OspA, $OspA_{(VNE)18}$ was susceptible to proteinase K treatment of intact cells, i.e. surface-exposed, while the periplasmic FlaB protein was protected (Fig. 6). A single +2 residue mutant , $OspA_{V18}$, behaved similarly (not shown). This confirmed that even a modified '+2 rule' does not apply to borrelial lipoproteins.

Figure 5. Amino-terminal sequences of *B. burgdorferi* lipoprotein mutants. *B. burgdorferi* strains are indicated in the first, recombinant plasmids in the second column. The lineage of mutated constructs is indicated by brackets to the left. *B. burgdorferi* B31 OspA and OppAIV lipopeptide sequences starting with the Cys at position +1 are marked by solid or dotted underlining, respectively. mRFP1 sequences are not underlined. Amino acid replacements are indicated in **bold**, deletions by a delta (Δ). The protein nomenclature is identical throughout the text: numericals following OspA and OppAIV indicate the length of N-terminal propeptides, i.e. starting with ^{f}Met. Mutants are designated in subscript by the introduced amino acid followed by their position in OspA and mRFP1, again with respect to the ^{f}Met. Sets of multiple amino acid changes are summarized by the peptide sequence in brackets followed by the position of the first mutated residue. Protein phenotypes are summarized as follows: asterisks (*) indicate surface localization of the expressed protein (see Figs. 6, 7 and 10); a double-dagger (‡) indicates that OspA28$_{(5A)18}$:mRFP1 is surface-exposed, but also forms protease-resistant aggregates (see text and Fig. 10A); a section sign (§) indicates that OspA$_{\Delta L24}$ localizes predominantly to the inner leaflet of the OM (Figs. 6 and 9); and daggers (†) denote unstable or toxic proteins (see text).


strain
plasmid
N-terminal peptide sequence
+1
protein
phenotype
B31 pRJS0999 C K Q N V S S L D E K N S V S V D L P OspA$_{wt}$ *
KU-1009 pRJS1009 C K Q N V S S L D E K N A S S E D V I OspA28:mRFP1 *
KU-1018 pRJS1012 C K Q N V S S L D A S S E D V I K E F OspA25:mRFP1 *
KU-1019 pRJS1013 C K Q N V S A S S E D V I K E F M R F OspA22:mRFP1 *
KU-1024 pRJS1015 C K Q N V A S S E D V I K E F M R F K OspA21:mRFP1 *
KU-1025 pRJS1016 C K Q N A S S E D V I K E F M R F K V OspA20:mRFP1
KU-1020 pRJS1014 C K Q A S S E D V I K E F M R F K V R OspA19:mRFP1
KU-1008 pRJS1009 C A S S E D V I K E F M R F K V R M E OspA17:mRFP1
KU-1028 pRJS1019 C K Q A A A S L D E K N A S S E D V I OspA28(3A)20:mRFP1 *
KU-1030 pRJS1021 C K Q N S S S L D E K N A S S E D V I OspA28S21:mRFP1 *
KU-1034 pRJS1025 C K Q N E S S L D E K N A S S E D V I OspA28E21:mRFP1 *
KU-1035 pRJS1026 C A A A A A S L D E K N A S S E D V I OspA28(5A)18:mRFP1 *‡
KU-1036 pRJS1027 C K Q A A A A A A E K N A S S E D V I OspA28(6A)20:mRFP1 *
KU-1037 pRJS1028 C K Q A A A S L D A A A A S S E D V I OspA28(3A)20/26:mRFP1 *
KU-1043 pRJS1034 C K Q N V S S E D E K N A S S E D V I OspA28E24:mRFP1 *
KU-1044 pRJS1035 C K Q N V E D L D E K N A S S E D V I OspA28(ED)22:mRFP1 *
KU-1039 pRJS1030 C K Q N V S S$_\Delta$D E K N A S S E D V I K OspA28ΔL24:mRFP1 *
KU-1052 pRJS1047 C S K M L A A L T R K T A S S E D V I OspArandom:mRFP1
KU-1032 pRJS1023 C K Q N A A A E D V I K E F M R F K V OspA20:mRFP1(2A)3
KU-1033 pRJS1024 C K Q N A S S A A V I K E F M R F K V OspA20:mRFP1(2A)5 *
KU-1040 pRJS1031 C K Q N A S S A D V I K E F M R F K V OspA20:mRFP1A5
KU-1041 pRJS1032 C K Q N A S S E A V I K E F M R F K V OspA20:mRFP1A6
KU-1046 pRJS1037 C K Q N V S S E D V I K E F M R F K V OspA20:mRFP1V2
KU-1022 pRJS1005 C V Q N V S S L D E K N S V S V D L P OspAV18 *
KU-1021 pRJS1004 C V N E V S S L D E K N S V S V D L P OspA(VNE)18 *
KU-1029 pRJS1020 C V N E S S S L D E K N S V S V D L P OspA(VNES)18 *
KU-1027 pRJS1018 C K Q A A A S L D E K N S V S V D L P OspA(3A)20 *
KU-1031 pRJS1022 C K Q N S S S L D E K N S V S V D L P OspAS21 *
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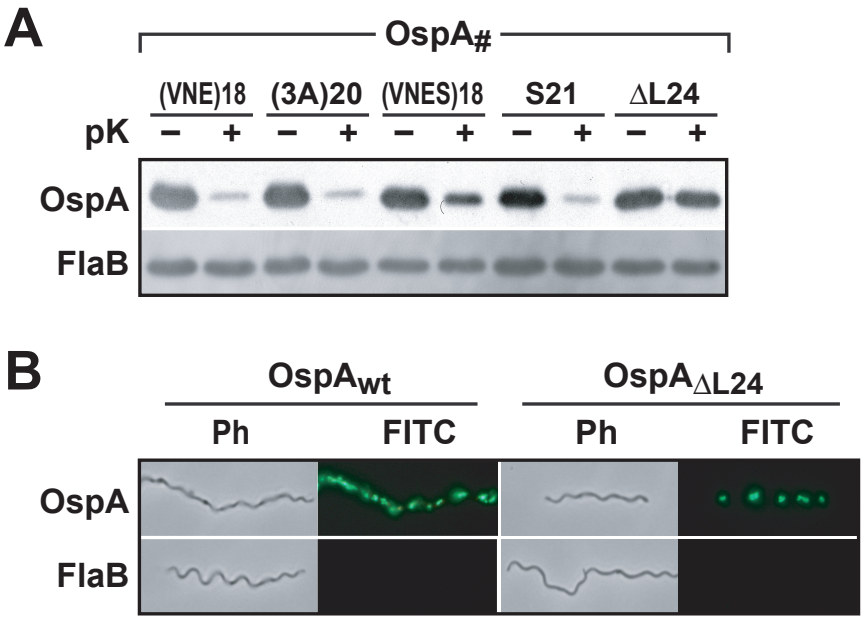
N/A pRJS1033 C K Q N V S S E D E K N S V S V D L P OspAE24 †
N/A pRJS1036 C K Q N V E D L D E K N S V S V D L P OspA(ED)22 †
B31 pRJS1000 C V N E S N R N K L V F K L N I G S E OppAIV$_{wt}$
KU-1010 pRJS1010 C V N E S N R N K L A S S E D V I K E OppAIV31:mRFP1 *

Figure 6. Localization of *B. burgdorferi* OspA mutants. (*A*) Lipoprotein surface exposure was assessed by incubating intact *B. burgdorferi* B313 cells expressing OspA mutants from recombinant plasmids (see Fig. 5 and text) with proteinase K (pK +) or control buffer (pK –), followed by Western immunoblotting with antibodies against OspA and the periplasmic flagellar protein FlaB. (*B*) Wild type and mutant OspA was localized by IFA microscopy of intact cells incubated with primary antibodies against OspA (w.t. surface control) and FlaB (periplasmic control) followed by a FITC-labelled secondary antibody. Micrographs were taken under phase contrast (Ph) and epifluorescence (FITC).


A
OspA#
(VNE)18
(3A)20
(VNES)18
S21
ΔL24
pK
– + – + – + – + – +
OspA
FlaB
B
OspAwt
OspAΔL24
Ph
FITC
Ph
FITC
OspA
FlaB

Five N-terminal OspA Residues Mediate Lipoprotein Surface Localization. Crystallographic data of OspA, OspC, VlsE, BbCRASP1 and Vsp1 *Borrelia* surface lipoproteins showed that their N-termini are unordered and likely provide flexible lipopeptide tethers to the structural proteins (Li et al., 1997; Zückert et al., 2001; Eicken et al., 2001; Kumaran et al., 2001; Eicken et al., 2002; Cordes et al., 2005; Lawson et al.,2006). We therefore expanded our search for *Borrelia* lipoprotein sorting signals from the +2 to +4 positions to span additional residues within these N-terminal tethers. To define the minimal targeting sequences while avoiding potential issues with mislocalized borrelial proteins, we generated fusions of N-terminal OspA lipopeptides to an inert reporter protein, monomeric red-fluorescent mRFP1 (Campbell et al., 2002). Our own preliminary studies (R.J. Schulze and W.R. Zückert, unpublished) as well as Chen *et al.* (Chen et al., 2005) showed that mRFP1, in contrast to green fluorescent protein (Feilmeier et al., 2000), is functional in the *E. coli* periplasm after Sec-dependent export. In a contemporaneous study, mRFP1 was also successfully fused to ActA, a C-terminally anchored surface protein of the gram-positive, monoderm *Listeria monocytogenes* (Rafelski and Theriot, 2005). We therefore inferred that mRFP1 may be a suitable marker for periplasmic and surface compartments of *B. burgdorferi*.

We first fused two N-terminal OspA peptides to mRFP1 by SOE PCR, cloned the chimeric genes into pBSV2 under P_{flaB} control and transformed *B. burgdorferi* B31-e2 cells. OspA17:mRFP1 included only the type II signal sequence and lipobox of OspA up to Cys^{17}, while OspA28:mRFP1 contained the OspA amino terminus up

to Asn^{28}, the last residue not confined in β-sheet secondary structure (Li et al., 1997). Both fusion proteins were detectable under epifluorescence and by Western immunoblotting (Fig. 7). Interestingly, the protein levels of OspA17:mRFP1 were about 3-fold lower than OspA28:mRFP1. Metabolic labelling with tritiated palmitic acid (Fig. 8A) and size analysis of immunoprecipitated proteins (Fig. 8B) showed that both OspA17- and OspA28:mRFP1 were properly lipidated and processed. Incubation of intact cells with proteinase K did not affect motility, i.e. the integrity of the periplasmic flagellar proteins such as FlaB, nor OspA17:mRFP1 fluorescence, but OspA28:mRFP1-expressing cells lost their fluorescence. OspA28:mRFP1 could be readily visualized under epifluorescence as a uniform red halo at the periphery of *B. burgdorferi* KU-1009 cells (Fig. 7B). These results indicated surface exposure of OspA28-, but not OspAC17:mRFP1, which was confirmed by Western immunoblotting (Fig. 7A) and indirect fluorescent-antibody (IFA) microscopy (Fig. 7C).

The above results suggested an OspA secretion signal between Cys^{17} and Asn^{28}. Therefore, we generated stepwise deletions of the OspA sequence in the OspA28:mRFP1 construct. All deletion constructs were expressed at the same level as OspA28:mRFP1 (Fig. 7A), properly processed and lipidated (Fig. 8). While deletion of 7 amino acids beyond Ser^{22} did not affect surface exposure, the additional deletion of Val^{21} led to subsurface localization of OspA20:mRFP1 (Fig. 7A). This indicated that the minimal secretion signal for lipidated OspA consists of only five N-terminal residues, including an apparently critical valine.

Figure 7. Localization of OspA-mRFP1 fusions expressed by *B. burgdorferi.* (*A*) Protease accessibility of N-terminal OspA-mRFP1 fusions expressed by recombinant *B. burgdorferi* B31-e2 cells (see Fig. 5 and text) was assessed by incubating intact cells with proteinase K (pK +) or control buffer (pK –), followed by epifluorescence microscopy and Western immunoblot analysis. Cells were observed under phase contrast (Ph) or epifluorescence using a Texas Red (TR) filter block. For Western immunoblotting, whole cell proteins fractionated by SDS-PAGE were probed with antibodies against mRFP1, OspA (w.t. surface control) and FlaB (periplasmic control). Note the decrease in molecular mass of the OspA28- to OspA17:mRFP1 fusion proteins. (*B*) Peripheral localization of surface-exposed OspAN28-mRFP1 visualized by epifluorescence microscopy of *B. burgdorferi* KU-1009 under 100 *X* magnification. The bar is 2 μm in length. (*C*) Indirect immunofluorescence micrographs confirming surface exposure of OspA28:mRFP1, but not OspA17: mRFP1. Intact cells were incubated with polyclonal antisera against mRFP1 and a FITC-conjugated Goat anti-Rabbit antibody and observed under phase contrast (Ph) and epifluorescence using FITC and Texas Red (TR) filters.

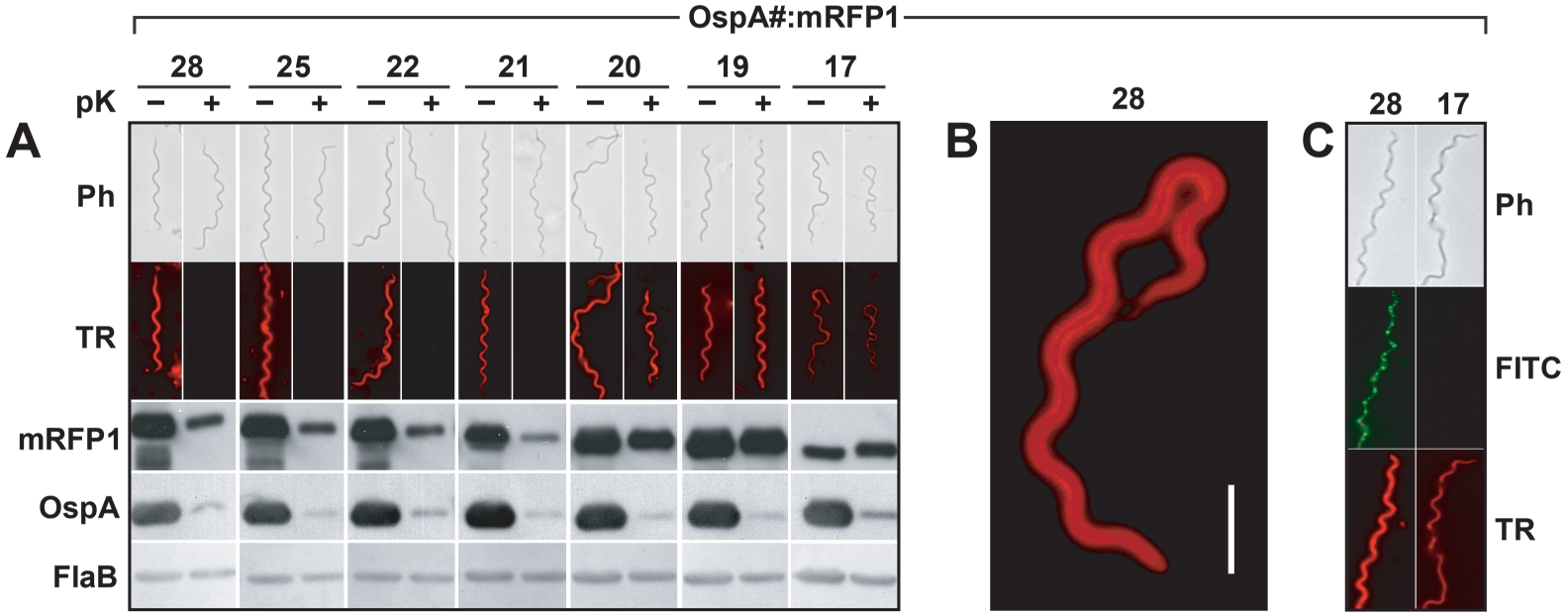
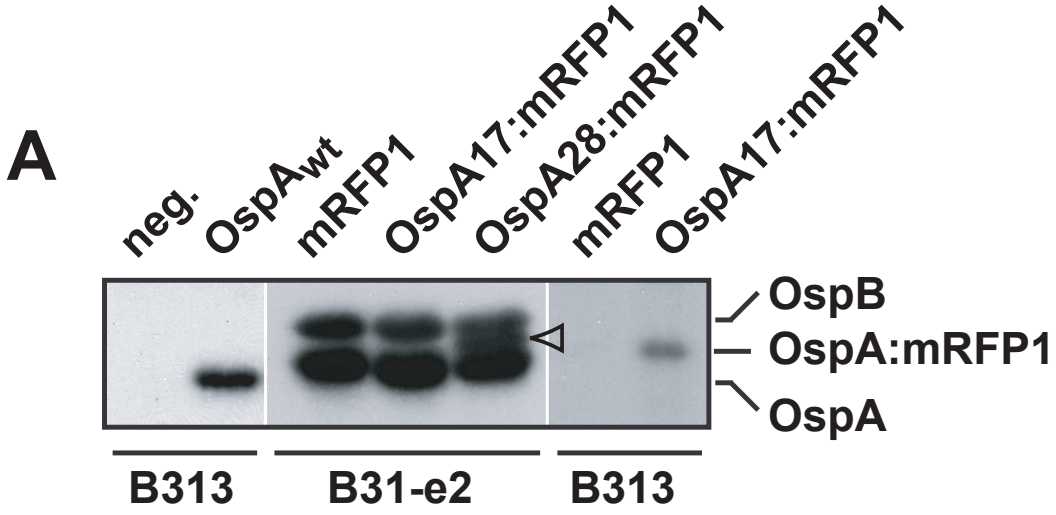

OspA#:mRFP1
28
25
22
21
20
19
17
pK
– + – + – + – + – + – + – +
A
Ph
TR
mRFP1
OspA
FlaB
B
28
C
28 17
Ph
FITC
TR

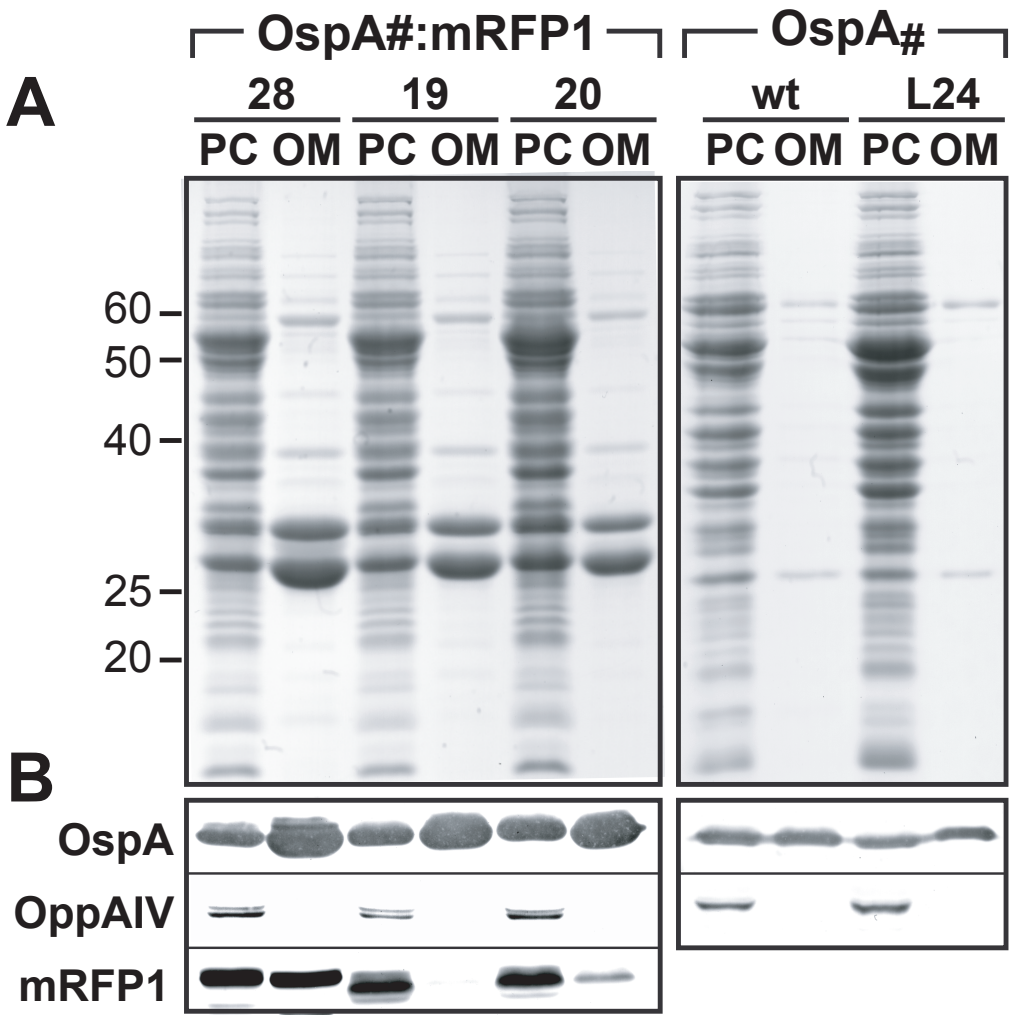
Figure 8. Lipidation and processing of lipoprotein mutants expressed by *B. burgdorferi.* (*A*) Lipidated OspA17- and OspA28:mRFP1 fusions were detected by radiography after metabolic labelling of *B. burgdorferi* B31-e2 and B313 harboring pRJS1008 and pRJS1009 (see Figs. 5 and 7) with tritiated palmitate. Controls were cells carrying pBSV2 (neg.) or pBSV2 derivatives for expression of w.t. OspA, ($OspA_{wt}$) or full-length non-lipidated mRFP1 (mRFP1). Note that OspA28:mRFP1, marked with an open arrowhead, migrates between the 31 kDa OspA and 34 kDa OspB band present in B31-e2; OspA17:mRFP1 comigrates with OspA, but is readily visible when expressed in the OspAB-deficient *B. burgdorferi* B313 background. (*B*) OspA17- to OspA28:mRFP1 fusions were immunoprecipitated with mRFP1 antisera from *B. burgdorferi* KU-1008 to KU-1009 and detected by Western immunoblotting. Note that lipoprotein sizes gradually increase from OspA17- to OspA28:mRFP1, indicating that the 1.7 kDa signal sequences of OspA17:mRFP1 as well as the other sub-surface fusions are cleaved by *B. burgdorferi* type II signal peptidase.


A
neg.
OspAwt
mRFP1
OspA17:mRFP1
OspA28:mRFP1
mRFP1
OspA17:mRFP1
OspB
OspA:mRFP1
OspA
B313
B31-e2
B313
B
OspA#:mRFP1
17 19 22 25 28
OspA:mRFP1

To assess whether the subsurface lipoprotein mutants were retained in the cytoplasmic membrane or in the inner leaflet of the outer membrane, we analysed membrane-associated proteins in protoplasmic cylinder (PC) and outer membrane vesicle (OMV) preparations (Fig. 9). *B. burgdorferi* OMVs were released by incubating cells in hypotonic citrate buffer, and PC and OMV fractions were separated on an isopycnic sucrose gradient. OspA28:mRFP1, like w.t. OspA, was found predominantly in the OMV fraction. W.t. OppAIV and subsurface OspA19– and OspA20:mRFP1 were detected predominantly in the PC fraction, demonstrating their retention within the IM (Fig. 9).

***Borrelia* Lipoproteins Are Secreted by Default.** We next probed the sequence specificity of *B. burgdorferi* secretion signals by performing an extensive site-directed mutagenesis of the OspA N-terminus (Figs 5, 6 and 10). First, we tested whether Val^{21} was indeed critical for OspA surface localization. Replacement of the hydrophobic Val^{21} in OspA28:mRFP1 with a polar serine ($OspA28_{S21}$:mRFP1) or negatively charged glutamate ($OspA28_{E21}$:mRFP1) did not alter lipoprotein localization, even though the same residues are present in identical positions in subsurface OspA19:mRFP1 and OppAIV, or OspA17:mRFP1, respectively (Fig. 7A). Val^{21} to Ser mutations in both w.t. OspA ($OspA_{S21}$) and $OspA_{(VNE)18}$ ($OspA_{(VNES)18}$) confirmed this finding (Fig. 6A). Furthermore, an Ala to Val mutation in subsurface OspA20:mRFP1 ($OspA20{:}mRFP1_{V2}$) did not lead to surface localization. These data

Figure 9. Membrane localization of lipoprotein mutants expressed by *B. burgdorferi*. (*A*) Coomassie-stained SDS-PAGE of *B. burgdorferi* B31-e2 cells expressing surface OspA28:mRFP1, subsurface OspA19- and OspA20:mRFP1 fusions (see Figs. 5 and 7) or B313 cells expressing w.t. OspA or $OspA_{\Delta L24}$ (see Figs. 5 and 6). PC, protoplasmic cylinder fraction; OM, outer membrane vesicle fraction enriched for OM proteins. Note that the PC fraction also contains OM proteins due to the partial separation of OMVs from protoplasmic cylinders by treatment of *Borrelia* cells with hypotonic citrate buffer (Skare et al., 1995). (*B*) Western immunoblots of PC and OMV fractions with antibodies against mRFP1 and controls OppAIV (PC) and OspA (OM).


A
OspA#:mRFP1
OspA#
28
19
20
wt
L24
PC OM PC OM PC OM
PC OM PC OM
60
50
40
25
20
B
OspA
OppAIV
mRFP1

indicated that Val^{21} by itself was neither sufficient nor essential for lipoprotein secretion.

Expanding our inquiry, we changed all residues from Lys^{18} to Asn^{28} in sets to alanines. All alanine mutants, i.e. $OspA28_{(3A)20}$: , $OspA_{(5A)18}$:, $OspA_{(6A)20}$:, and $OspA_{(3A)20/26}$:mRFP1, were protease-sensitive, indicating that they were surface-exposed (Fig. 10). $OspA_{(5A)18}$:mRFP1 for unknown reasons produced protease-resistant mRFP1 aggregates. This suggested that, in the absence of a specific IM retention signal, borrelial lipoproteins are deployed to the surface by default. To test for an N-terminal retention signal, we fused mRFP1 to the N-terminus of OppAIV, our model subsurface lipoprotein. As Val^{32} is predicted by the PSIPRED algorithm (Jones, 1999) to be the first structurally confined OppAIV residue, we fused the first 31 amino acids of OppAIV to mRFP1. Surprisingly, the resulting OppAIV31:mRFP1 fusion was surface exposed (Fig. 10C). This further supported our hypothesis of a default lipoprotein secretion pathway, and suggested that IM retention signals for OppAIV are confined to the structural protein.

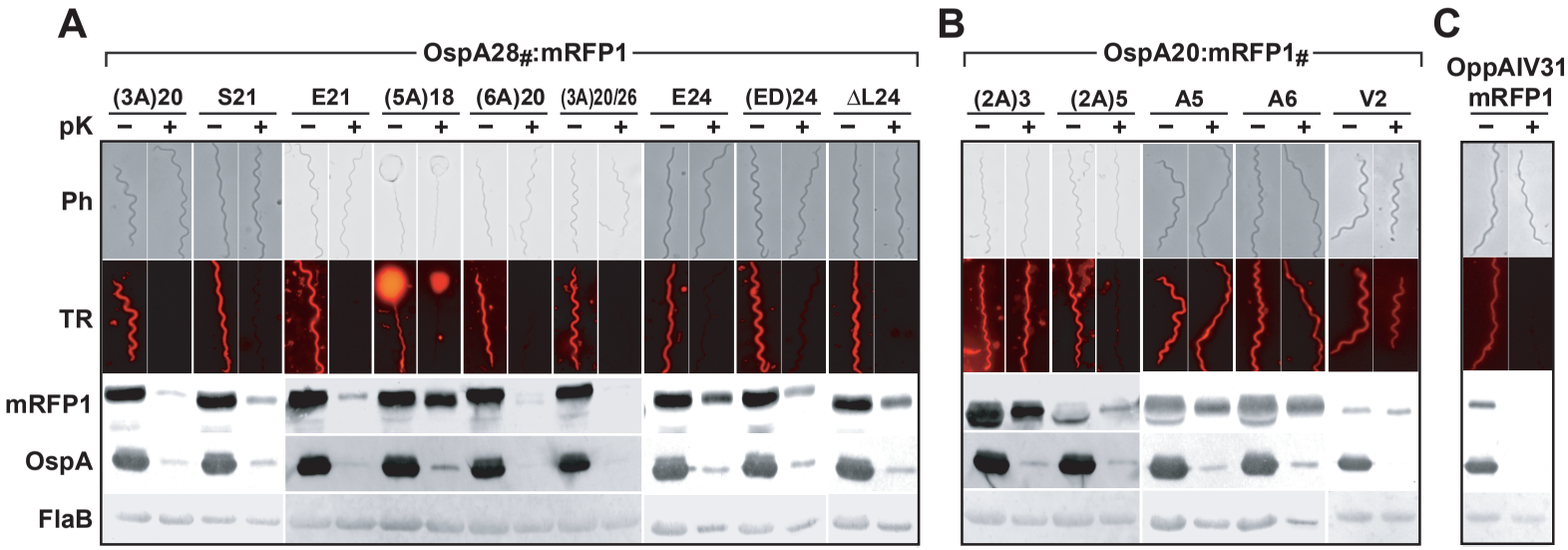
Negatively Charged Residues Can Act as Specific N-terminal Retention Signals. The lack of an OppAIV N-terminal IM retention signal led us to ask why OspA20:mRFP1 was retained in the IM. As described above, substitutions of OspA Val^{21} did not change the fusion proteins surface localization, and we speculated that residues in the immediate N-terminus of mRFP1 (Ala^2-Ser^3-Ser^4-Glu^5-Asp^6), in particular the two negatively charged Glu and Asp residues, might inadvertently

provide a retention signal for OspA20:mRFP1 and the shorter OspA:mRFP1 fusions. We therefore substituted both serines and the following Glu and Asp residues of OspA20:mRFP1 in sets to alanines. Substitutions of Ser^{3}-Ser^{4} to Ala-Ala (OspA20:mRFP1$_{(2A)3}$), Glu^{5} to Ala (OspA20:mRFP1$_{A5}$) and Asp^{6} to Ala (OspA20:mRFP1$_{A6}$) did not alter protein localization, while the substitution of Glu^{5}-Asp^{6} to Ala-Ala (OspA20:mRFP1$_{(2A)5}$) led to the release of the fusion protein from the IM and its display on the bacterial surface (Fig. 10B). This suggested that negatively charged residues within the first nine N-terminal amino acids of the mature lipoprotein can retain lipoproteins in the *B. burgdorferi* periplasm.

N-terminal Amino Acid Sequence Determinants of OspA surface exposure.

Asp^{25} and Glu^{26} of OspA are in positions +9 and +10, i.e. one residue removed from where they potentially could serve as a retention signal. To test whether a positional effect of negative charges alone is sufficient to retain OspA in the IM, we mutated both w.t. OspA or OspA28:mRFP1 proteins by either deleting Leu^{24} or replacing it with Glu, or by replacing Ser^{22}-Ser^{23} with Glu-Asp. All three OspA-mRFP1 fusion mutants, OspA28$_{\Delta L24}$-, OspA28$_{E24}$-, and OspA28$_{(ED)22}$:mRFP1 were expressed by KU-1039, KU-1043 and KU-1044, respectively, and remained surface-exposed (Fig. 10A). No *B. burgdorferi* transformants expressing the OspA Leu^{24} to Glu (OspA$_{E24}$) and Ser^{22}-Ser^{23} to Glu-Asp (OspA$_{ED22}$) were recovered, indicating that the introduction of additional negative charges into the N-terminus of OspA may be detrimental to the *B. burgdorferi* cell. The deletion of Leu^{24} in w.t. OspA (OspA$_{\Delta L24}$),

Figure 10. Localization of OspA and OppAIV lipopeptide-mRFP1 fusion mutants expressed by *B. burgdorferi*. Protease accessibility was assessed by in situ surface proteolysis of intact *B. burgdorferi* B31-e2 cells expressing recombinant proteins (see Fig. 5 and text). Cells were incubation with proteinase K (pK +) or control buffer (pK –), followed by epifluorescence microscopy and Western immunoblot analysis. Cells were observed under phase contrast (Ph) or epifluorescence using a Texas Red (TR) filter block. For Western immunoblotting, whole cell proteins fractionated by SDS-PAGE were probed with antibodies against mRFP1, OspA (w.t. surface control) and FlaB (periplasmic control). (*A*) Protease accessibility of OspA28-mRFP1 mutants. Note that OspA28$_{(5A)18}$:mRFP1 appears succeptible to protease, i.e. surface-exposed, along the borrelial cell, but forms polar protease-resistant aggregates. (*B*) Localization of OspA20-mRFP1 mutants. Note that only the OspA20:mRFP1$_{(2A)5}$ mutant is released from the IM to the surface. (*C*) Protease accessibility of OppAIV31: mRFP1.


A
OspA28#:mRFP1
(3A)20
S21
E21
(5A)18
(6A)20
(3A)20/26
E24
(ED)24
ΔL24
pK
Ph
TR
mRFP1
OspA
FlaB
B
OspA20:mRFP1#
(2A)3
(2A)5
A5
A6
V2
C
OppAIV31:
mRFP1

however, was tolerated. Although $OspA_{\Delta L24}$ was still detected on the cell surface by immunofluorescence microscopy (Fig. 6B), densitometry on Western immunoblots (Fig. 6A) and on Coomassie-stained SDS-PAGE gels (not shown) indicated that about two thirds of the mutant OspA was protease-resistant. In contrast to the subsurface OspA:mRFP1 mutants, which associated predominantly with the IM, $OspA_{\Delta L24}$ was found in the OM at w.t. OspA levels (Fig. 9). W.t. lipidated OspA and $OspA_{\Delta L24}$ extracted from *B. burgdorferi* membranes by Triton X-114 detergent were equally protease-sensitive (not shown), refuting the already remote possibility that deletion of Leu^{24} would render the numerous aliphatic and aromatic residues throughout the OspA molecule intrinsically resistant to cleavage by proteinase K (Keil, 1992). This indicated that $OspA_{\Delta L24}$ is released from the IM, but accumulates in the inner leaflet of the OM because of a defect in efficient translocation through the OM.

Discussion

In this study, we analyzed the peptide sequences targeting spirochaetal lipoproteins to different cellular compartments by using two model surface and subsurface *Borrelia* lipoproteins, *B. burgdorferi* OspA and OppAIV. We demonstrated that the '+2 rule' established for *E. coli* IM and OM lipoproteins does not apply to borrelial lipoproteins. To the contrary, fusions of N-terminal tether lipopeptides of both OspA and OppAIV mediated the secretion and surface display of an inert fluorescent reporter protein, mRFP1, and an extensive mutagenesis of the OspA lipopeptide

tether showed that secretion is largely independent of peptide sequence. Supporting the latter, the N-termini of mature surface lipoproteins are not conserved beyond orthologous or paralogous lipoprotein protein families such as the OspC/Vsps or OspEF/Erps (Zückert et al., 2001; Stevenson et al., 1996; Casjens et al., 2000; Fraser et al., 1997). This indicates that in absence of protein-specific retention signals, the default pathway leads to the secretion of lipoproteins to the bacterial surface, and suggests that tether peptides of w.t. subsurface *Borrelia* lipoproteins do not *per se* contain signals that restrict the proteins to the IM.

Secretion also appears independent of overall lipoprotein secondary structure since both α-helical and β-sheet lipoproteins, e.g. OspC and OspA (Eicken *et al*., 2001; Kumaran *et al*., 2001; Li *et al*., 1997), are found on the spirochaetal surface. Furthermore, the lipoproteins N-terminal tether peptides are neither structurally constrained in protein crystals nor are they predicted to form secondary structures, including transmembrane domains. This is in contrast to surface lipoproteins of other diderm bacteria, which require structural information for their secretion. A well-studied example is PulA, a 116-kDa amylolytic enzyme secreted and anchored to the surface of the Gram-negative bacterium *Klebsiella oxytoca* (d'Enfert *et al*., 1987). PulA is exported by the main terminal branch of the general secretory pathway, i.e. a prototypical type II secretion system (Pugsley, 1993). The PulA secretion signal resides within the tertiary structure of the protein in multiple, non-adjacent regions (Francetic and Pugsley, 2005; Sauvonnet and Pugsley, 1996). In the absence of a type II secretion system, the penultimate Asp in PulA functions as IM retention/Lol

avoidance signal (Pugsley *et al*., 1990). Other surface lipoproteins, such as the *Bordetella pertussis* SphB1 subtilisin (Coutte *et al*., 2003) or the *Neisseria meningitidis* NalP serine protease (van Ulsen *et al*., 2003), are autotransported via a C-terminal porin-like translocator domain (Oomen *et al*., 2004).

The mechanisms leading to the periplasmic retention of OppAIV and other subsurface *Borrelia* lipoproteins remain to be determined. For *E. coli* lipoproteins, it has been suggested that IM retention/Lol avoidance involves the interaction of Asp_{+2} and adjacent residues with the zwitterionic phosphatidylethanolamine in the IM bilayer (Hara *et al*., 2003). In contrast to other bacteria, *Borrelia* lipid bilayers contain only phosphatidylglycerol and phosphatidylcholine (Belisle *et al*., 1994; Hossain *et al*., 2001), and it remains to be determined whether retention of IM *Borrelia* lipoproteins can be mediated by the interactions of N-terminal residues with the zwitterionic phosphatidylcholine. Otherwise, lipoproteins might be retained in the IM by other lipid-protein or protein-protein interactions.

Negative charges within the lipoprotein tethers can disrupt lipoprotein secretion, but the underlying mechanisms may vary depending on peptide context. On one hand, the mRFP1 $Glu_{+5}Asp_{+6}$ IM retention signal of OspA19:mRFP1 bears similarity to the Asp_{+2} IM retention/Lol avoidance signal of *E. coli* lipoproteins, which can be further enhanced by additional negatively charged residues in the +3 position (Robichon *et al*., 2003; Masuda *et al*., 2002; Terada *et al*., 2001). Based on the determined structure of w.t. *E. coli* Lpp, the N-terminal tether of the three-stranded coiled coil consists of only two residues, Cys_{+1} and Ser_{+2} (Shu *et al*., 2000).

Therefore, the +2 and +3 residues affecting the efficiency of sorting (Terada *et al*., 2001) are proximal to the structurally confined Lpp N-terminus. Likewise, Asp_{+6} is the predicted first structurally confined N-terminal residue of mRFP1 (Campbell *et al*., 2002), and the OspA19:$mRFP1_{(2A)5}$ mutant may be released from the IM to the surface because of its altered tether-to-β-sheet transition. On the other hand, $OspA_{\Delta L24}$ is efficiently released from the IM, but is defective in translocation through the OM. If the $OspA_{\Delta L24}$ phenotype is solely due to the shifted position of negative charges within the OspA tether, identically positioned negatively charged dipeptides in the surface-localized $OspA28_{E24-}$ and $OspA28_{\Delta L24}$:mRFP1 fusions or the w.t. OspE/Erp proteins (Stevenson *et al*., 1996) would have to be somehow neutralized by other residues. As precedent, the *E. coli* Asp_{+2} lipoprotein retention signal can be at least partially overcome by residues in the immediately adjacent +3 position (Gennity and Inouye, 1991; Terada *et al*., 2001). Alternatively, a minimum tether length may be required for efficient secretion. The mature N-termini of OspA and other *Borrelia* surface lipoproteins show no evidence of transmembrane domains, and lipidated recombinant proteins or fluorescent fatty acids insert spontaneously into the lipopolysaccharide-deficient apical leaflet of the *Borrelia* OM (Bunikis *et al.*, 2001; Cox and Radolf, 2001). Therefore, it can be all but ruled out that *Borrelia* surface lipoproteins are anchored via their triacyl moiety in the periplasmic leaflet and that N-terminal mutations simply affect the spanning of the OM.

The borrelial lipoprotein secretion machinery likely contains components common to diderm bacteria as well as spirochaete-specific protein complexes.

Orthologs of all essential components of the Sec translocase complex are present in *Borrelia* and all other spirochaetal genomes identified so far, and the same is true for the three enzymes required for lipoprotein modification, Lgt prolipoprotein diacylglyceryl transferase, Lsp signal II peptidase and Lnt apolipoprotein N-acyltransferase. Yet, with the exception of potential type II secretion and twin-arginine translocation (Tat) orthologs in *Leptospira*, further Sec-dependent or -independent bacterial protein secretion pathways appear to be absent in spirochaetes (Haake, 2000; Fraser *et al*., 1998; Seshadri *et al*., 2004; Nascimento *et al*., 2004). Together with the here described secretion-facilitating N-terminal peptide sequences, this virtually precludes the involvement of the two known lipoprotein secretion pathways. Rather, the above-described potential analogies in OspA and Lpp sorting signal sequences may further argue for an involvement of a Lol-like pathway. Intriguingly, orthologs of the IM ABC transporter-like sortase complex LolCDE (Yakushi *et al*., 2000), the periplasmic lipoprotein-binding chaperone LolA (Yokota *et al*., 1999), but not the OM lipoprotein receptor LolB (Yokota *et al*., 1999), have been identified in the *B. burgdorferi* genome. The lack of LolB in *Borrelia* might reflect the periplasmic or surface localization of *Borrelia* and *E. coli* major OM lipoproteins to different leaflets. We therefore hypothesize that the lipoprotein transport pathway in *Borrelia* consists of LolCDE and LolA orthologs, but diverges from that of other diderm bacteria by utilizing a sofar unidentified module to facilitate translocation of surface lipoproteins through the OM.

mRFP1 (Campbell *et al*., 2002) is currently the only fluorescent marker compatible with Sec-dependent export in diderm organisms, as the most commonly used fluorescent protein GFP is only periplasmically active when exported as already folded protein via the Tat pathway (Thomas *et al*., 2001; Feilmeier *et al*., 2000). The results of this study expand the use of fluorescent proteins from the cyto- and periplasmic compartments to the bacterial surface of diderm bacteria. mRFP1 and its recently described yellow-to-red fluorescent derivatives (Shaner *et al*., 2004) certainly have the potential to become valuable tools in further analyses of protein secretion in a variety of bacterial cells, including *B. burgdorferi*. In particular, they will permit the use and recovery of viable cells in fluorescence-based screening approaches selecting for protein surface and subsurface localization.

Experimental procedures

Bacterial strains and growth conditions. *B. burgdorferi* B31-e2 (Babb et al., 2004) and B313 (Sadziene et al., 1993) are clones of type strain B31 (ATCC 35210). B31-e2 was generously provided by B. Stevenson (University of Kentucky, Lexington, KY) and contains plasmids cp26, cp32-1, cp32-3, cp32-4, lp17, lp38, and lp54 (Babb et al., 2004). B313 carries cp26, cp32-1, cp32-2/7, cp32-3 and lp17 (Zückert et al., 1999; Zückert et al., 2004). *B. burgdorferi* were cultured in liquid or solid BSK-II medium at 34°C under 5% CO_2 (Barbour, 1984). *E. coli* strains TOP10 (Invitrogen, Carlsbad, CA) and XL10-Gold (Stratagene) were used for recombinant plasmid construction and propagation and grown in Luria Bertani Lennox broth (LB) or on LB agar (Difco).

Lipoprotein Fusion and Point Mutants. The recombinant plasmids generated and used in this study (see Fig. 5 and Table 1, which is published as supporting information) are derivatives of pBSV2 (Stewart et al., 2001), which replicates autonomously in both *E. coli* and *B. burgdorferi* and confers resistance to kanamycin. In all plasmid constructs, lipoprotein expression is driven by the constitutive *B. burgdorferi* flagellin *flaB* promoter (P_{flaB}). Transcriptional and translational fusions were generated by sequence overlap extension (SOE) PCR (Ho et al., 1989) using Platinum *Pfx* polymerase (Invitrogen, Carlsbad, CA) and the oligonucleotides listed in Table 2 (which is published as supporting information on the PNAS website). Fusion PCR amplicons were digested with *Bam*HI/*Xba*I or *Bam*HI/*Sph*I for OspA or

Table 1. Strains and plasmids used in this study.

B. burgdorferi Strain	Plasmid	Description	Comments †
KU-0998	pRJS0999	pBSV2.PflaBospA	in B313 background
KU-0999	pRJS1000	pBSV2.PflaBoppAIV	in B31-82 background
KU-1021	pRJS1004	pRJS0999 with (VNE)18 mutation	in B313 background
KU-1022	pRJS1005	pRJS0999 with V18 mutation	in B313 background
KU-1008	pRJS1008	pBSV2.PflaBospA17:mRFP1 fusion	
KU-1009	pRJS1009	pBSV2.PflaBospA28:mRFP1 fusion	
KU-1010	pRJS1010	pBSV2.PflaBoppAIV31:mRFP1 fusion	
KU-1018	pRJS1012	pBSV2.PflaBospA25:mRFP1 fusion	
KU-1019	pRJS1013	pBSV2.PflaBospA22:mRFP1 fusion	
KU-1020	pRJS1014	pBSV2.PflaBospA19:mRFP1 fusion	
KU-1024	pRJS1015	pBSV2.PflaBospA21:mRFP1 fusion	
KU-1025	pRJS1016	pBSV2.PflaBospA20:mRFP1 fusion	
KU-1027	pRJS1018	pRJS0999 with N20V21S22 to AAA mutation	in B313 background
KU-1028	pRJS1019	pRJS1009 with N20V212S22 to AAA mutation	
KU-1029	pRJS1020	pRJS0999 with KQNV to VNES mutation	in B313 background
KU-1030	pRJS1021	pRJS1009 with V21S point mutation	
KU-1031	pRJS1022	pRJS0999 with V21S point mutation	in B313 background
KU-1032	pRJS1023	pRJS1016 with RFP S3S4 to AA mutation	
KU-1033	pRJS1024	pRJS1016 with RFP E5D6 to AA mutation	
KU-1034	pRJS1025	pRJS1009 with V21E point mutation	
KU-1035	pRJS1026	pRJS1019 with K18Q19 to AA mutation	
KU-1036	pRJS1027	pRJS1019 with S23L24D25 to AAA mutation	
KU-1037	pRJS1028	pRJS1019 with E26K27N28 to AAA mutation	
KU-1038	pRJS1029	pRJS0999 with L24 deletion	in B313 background
KU-1039	pRJS1030	pRJS1009 with L24 deletion	
KU-1040	pRJS1031	pRJS1016 with RFP E5A mutation	
KU-1041	pRJS1032	pRJS1016 with RFP D6A mutation	
KU-1042	pRJS1033	pRJS0999 with L24E mutation	in B313 background
KU-1043	pRJS1034	pRJS1009 with L24E mutation	
KU-1044	pRJS1035	pRJS1009 with S22S23 to ED mutation	
KU-1045	pRJS1036	pRJS0999 with S22S23 to ED mutation	in B313 background
KU-1046	pRJS1037	pRJS1016 with A21V point mutation	
KU-1047	pRJS1038	pRJS1030 with V21A point mutation	
KU-1048	pRJS1039	pRJS1029 with V21A point mutation	in B313 background

*Amino acids are indicated in single letter code with their position in the full-length preprotein (see also text).
† strains are in *B. burgdorferi* B31-e2 background unless noted otherwise.

Table 2. Oligonucleotides used in this study.

Name	Sequence	Description
BamPflaB-fwd	5' **CGGGATCC**TGTCTGTCGCCTCTTG 3'	Forward flanking primer 5' of *flaB* promoter (5' *Bam*HI extension)
XbaOspA-rev	5' **GCTCTAGA**TTATTTTAAAGCGTTTTTAATTTC 3'	Reverse flanking primer 3' of OspA (3' *Xba*I extension)
SphmRFP-rev	5' **CTTGCATGCTCA**TTAGGCGCCGGTGGAGTG 3'	Reverse flanking primer 3' of mRFP1 (3' *Sph*I extension)
1003-fwd	5' **GTAATAGAAACAAATTG**AGCGTTTCAGTAGATTTGC 3'	Forward SOE primer for creating pRJS1003
1003-rev	5' **GCAAATCTACTGAAACGCT**CAATTTGTTTCTATTAC 3'	Reverse SOE primer for creating pRJS1003
1004-fwd	5' GCCTTAATAGCATGT**GTAAATGAA**GTTAGCAGCCTTGACGAG 3'	Forward mutagenic primer for creating pRJS1004 from pBSV2:PflaBospA
1004-rev	5' CTCGTCAAGGCTGCTAAC**TTCATTTAC**ACATGCTATTAAGGC 3'	Reverse mutagenic primer for creating pRJS1004 from pBSV2:PflaBospA
1005-fwd	5' GCCTTAATAGCATGT**GTAC**AAAATGTTAGC 3'	Forward mutagenic primer for creating pRJS1005 from pBSV2:PflaBospA
1005-rev	5' CAAGGCTGCTAACATTTTG**TAC**ACATGCTATTAAGGC 3'	Reverse mutagenic primer for creating pRJS1005 from pBSV2:PflaBospA
1008-fwd	5' **CCTTAATAGCATGT**GCCTCCTCCGAGGACGTCATC 3'	Forward SOE primer for creating pRJS1008
1008-rev	5' **GATGACGTCCTCGGAGGAGGC**ACATGCTATTAAGGC 3'	Reverse SOE primer for creating pRJS1008
1009-fwd	5' **CTTGACGAGAAAAAC**GCCTCCTCCGAGGACGTCATC 3'	Forward SOE primer for creating pRJS1009
1009-rev	5' **GATGACGTCCTCGGAGGAGGC**GTTTTTCTCGTCAAG 3'	Reverse SOE primer for creating pRJS1009
1012-fwd	5' GTTAGCAGCCTTGAC|GCCTCCTCCGAGGACGTCATC 3'	Forward mutagenic primer for creating pRJS1012 from pRJS1009
1012-rev	5' ATGACGTCCTCGGAGGAGGC|GTCAAGGCTGCTAAC 3'	Reverse mutagenic primer for creating pRJS1012 from pRJS1009
1013-fwd	5' ATGTAAGCAAAATGTTAGC|GCCTCCTCCGAGGAC 3'	Forward mutagenic primer for creating pRJS1013 from pRJS1012
1013-rev	5' GTCCTCGGAGGAGGC|GCTAACATTTTGCTTACAT 3'	Reverse mutagenic primer for creating pRJS1013 from pRJS1012
1014-fwd	5' ATTAGCCTTAATAGCATGT**AAGCAA**GCCTCCTCCGAGGAC 3'	Forward mutagenic primer for creating pRJS1014 from pRJS1008
1014-rev	5' GTCCTCGGAGGAGGC**TTGCTT**ACATGCTATTAAGGCTAAT 3'	Reverse mutagenic primer for creating pRJS1014 from pRJS1008
1015-fwd	5' ATGTAAGCAAAATGTT|GCCTCCTCCGAGGACGTCATC 3'	Forward mutagenic primer for creating pRJS1015 from pRJS1013
1015-rev	5' CCTCGGAGGAGGC|AACATTTTGCTTACATGCTATTAAGG 3'	Reverse mutagenic primer for creating pRJS1015 from pRJS1013
1016-fwd	5' AGCATGTAAGCAA**AAT**GCCTCCTCCGAGGACGTCATC 3'	Forward mutagenic primer for creating pRJS1016 from pRJS1014
1016-rev	5' CTCGGAGGAGGC**ATT**TTGCTTACATGCTATTAAGG 3'	Reverse mutagenic primer for creating pRJS1016 from pRJS1014
1018-fwd	5' TAATAGCATGTAAGCAA**GCAGCAGCA**AGCCTTGACGAG 3'	Forward mutagenic primer for creating pRJS1018 and pRJS1019 from pBSV2:PflaBospA or pRJS1009
1018-rev	5' CTCGTCAAGGCT**TGCTGCTGC**TTGCTTACATGCTATTAAGG 3'	Reverse mutagenic primer for creating pRJS1018 and pRJS1019 from pBSV2:PflaBospA or pRJS1009
1021-fwd	5' AGCCTTAATAGCATGTAAGCAAAAT**AGC**AGCAGCCTTGAC 3'	Forward mutagenic primer for creating pRJS1021 and pRJS1022 from pBSV2:PflaBospA or pRJS1009
1021-rev	5' TCGTCAAGGCTGCT**GCT**ATTTTGCTTACATGCTATTAAGG 3'	Reverse mutagenic primer for creating pRJS1021 and pRJS1022 from pBSV2:PflaBospA or pRJS1009

Table 2. Oligonucleotides used in this study (cont.)

Name	Sequence	Description
1023-fwd	5' GCATGTAAGCAAAATGCC**GCCGCC**GAGGACGTCATCAAGG 3'	Forward mutagenic primer for creating pRJS1023 from pRJS1016
1023-rev	5' CCTTGATGACGTCCTC**GGCGGC**GGCATTTTGCTTACATGC 3'	Reverse mutagenic primer for creating pRJS1023 from pRJS1016
1024-fwd	5' AAATGCCTCCTCC**GCCGCC**GTCATCAAGGAGTTCATGC 3'	Forward mutagenic primer for creating pRJS1024 from pRJS1016
1024-rev	5' ACTCCTTGATGAC**GGCGGC**GGAGGAGGCATTTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1024 from pRJS1016
1025-fwd	5' TAATAGCATGTAAGCAAAAT**GAG**AGCAGCCTTGAC 3'	Forward mutagenic primer for creating pRJS1025 from pRJS1009
1025-rev	5' GTCAAGGCTGCT**CTC**ATTTTGCTTACATGCTATTAAGG 3'	Reverse mutagenic primer for creating pRJS1025 from pRJS1009
1026-fwd	5' CTTAATAGCATGT**GCCGCC**GCAGCAGCAAGCCTTGAC 3'	Forward mutagenic primer for creating pRJS1026 from pRJS1019
1026-rev	5' GCTTGCTGCTGC**GGCGGC**ACATGCTATTAAGGC 3'	Reverse mutagenic primer for creating pRJS1026 from pRJS1019
1027-fwd	5' AAGCAAGCAGCAGCA**GCCGCTGCG**GAGAAAAACG 3'	Forward mutagenic primer for creating pRJS1027 from pRJS1019
1027-rev	5' GCGTTTTTCTC**CGCAGCGGC**TGCTGCTGCTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1027 from pRJS1019
1028-fwd	5' CAGCAAGCCTTGAC**GCCGCTGCA**GCCTCCTCCGAG 3'	Forward mutagenic primer for creating pRJS1028 from pRJS1019
1028-rev	5' CTCGGAGGAGGC**TGCAGCGGC**GTCAAGGCTTGCTGC 3'	Reverse mutagenic primer for creating pRJS1028 from pRJS1019
1029-fwd	5' TGTAAGCAAAATGTTAGCAGC|GACGAGAAAAAC 3'	Forward mutagenic primer for creating pRJS1029 and pRJS1030 from pBSV2:PflaBospA or pRJS1009
1029-rev	5' GTTTTTCTCGTC|GCTGCTAACATTTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1029 and pRJS1030 from pBSV2:PflaBospA or pRJS1009
1031-fwd	5' TAAGCAAAATGCCTCCTCC**GCC**GACGTCATCAAG 3'	Forward mutagenic primer for creating pRJS1031 from pRJS1016
1031-rev	5' CTTGATGACGTC**GGC**GGAGGAGGCATTTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1031 from pRJS1016
1032-fwd	5' AAATGCCTCCTCCGAG**GCC**GTCATCAAGGAGTTC 3'	Forward mutagenic primer for creating pRJS1032 from pRJS1016
1032-rev	5' AACTCCTTGATGAC**GGC**CTCGGAGGAGGCATTTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1032 from pRJS1016
1033-fwd	5' TGTAAGCAAAATGTTAGCAGCGAGGAC**GAG**AAAAAC 3'	Forward mutagenic primer for creating pRJS1033 and pRJS1034 from pBSV2:PflaBospA or pRJS1009
1033-rev	5' GTTTTT**CTC**GTCCTCGCTGCTAACATTTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1033 and pRJS1034 from pBSV2:PflaBospA or pRJS1009
1035-fwd	5' TGTAAGCAAAATGTT**GAGGAC**CTTGACGAGAAAAAC 3'	Forward mutagenic primer for creating pRJS1035 and pRJS1036 from pBSV2:PflaBospA or pRJS1009
1035-rev	5' GTTTTTCTCGTCAAG**GTCCTC**AACATTTTGCTTAC 3'	Reverse mutagenic primer for creating pRJS1035 and pRJS1036 from pBSV2:PflaBospA or pRJS1009

*Underlined nucleotides are restriction sites used for cloning. Bold nucleotides are mutated sequence or sequence not complementary to the template. Vertical lines (|) represent nucleotides deleted from the template.

mRFP1 constructs, respectively, and ligated into pBSV2. Point mutations were generated by either SOE PCR or using the QuikChange II XL site-directed mutagenesis kit (Stratagene). All constructs were checked for mutations by sequencing (ABI BigDye Terminator cycle sequencing kit and ABI 3130*xl* Genetic Analyzer, Biotechnology Core Facility, KUMC). *B. burgdorferi* cells were transformed by electroporation using 1-5 μg of plasmid DNA using established protocols (Stewart et al., 2001). Transformants were selected in solid BSK-II containing 200 μg/ml kanamycin, and at least two independent clones were expanded in selective liquid BSK-II. Plasmid profiles were determined by PCR using plasmid-specific oligonucleotide primers (Purser and Norris, 2000; Labandeira-Rey and Skare, 2001). The resulting recombinant strains are listed in Table 1 (see supporting information).

Protein Gel Electrophoresis and Immunoblot analysis. Proteins were separated by sodium dodecyl sulfate-12% polyacrylamide electrophoresis (SDS-PAGE) and visualized by Coomassie blue staining. For immunoblots, proteins were electrophoretically transferred to nitrocellulose membranes (Immobilon-NC, Millipore) using a Transblot-SD Semi-Dry Transfer Cell (Bio-Rad) as described (Zückert et al., 1999). Membranes were blocked and incubated with antibodies in 5% dry milk, 20 mM Tris-500 mM NaCl, 0.05% Tween 20 as described (Zückert et al., 2004). Antibodies used were anti-mRFP1 rabbit polyclonal antiserum (1:1,000 dilution, a gift from P. Viollier, Case Western Reserve University, Cleveland, OH),

anti-OppAIV rabbit polyclonal antiserum (1:100 dilution, a gift from P.A Rosa, NIH/NIAID Rocky Mountain Laboratories, Hamilton, MT) (Bono et al., 1998), or monoclonal antibodies against *B. burgdorferi* OspA (1:25 dilution, H5332) (Barbour et al., 1983) and FlaB (1:25 dilution, H9724) (Barbour et al., 1986). Secondary antibodies were alkaline phosphatase-conjugated goat-anti-rabbit IgG (H+L) or goat-anti-mouse IgG (H+L) (Bio-Rad). Alkaline phosphatase substrates were 1-Step NBT/NCIB (Pierce) for colorimetric and CDP-Star (Amersham Biosciences) for chemiluminescent detection.

Protease and Antibody Accessibility Assays. Spirochaetes were harvested, washed and resuspended in phosphate-buffered saline containing 5mM $MgCl_2$ (PBS+Mg) as described (Barbour, 1984). To assess protein surface exposure by protease accessibility, intact *B. burgdorferi* cells were treated *in situ* with 200 µg/ml proteinase K (Invitrogen) as described (Bunikis and Barbour, 1999). As a control, cells were sonicated using a Branson Sonifier cell disruptor prior to protease treatment.

Antibody accessibility was assessed by indirect immunofluorescence (IFA) as described (Zückert et al., 1999). Spirochaetes were resuspended in PBS+Mg supplemented with 2% bovine serum albumin (BSA) and incubated with the primary antibodies detailed above. The secondary antibodies were FITC-labeled goat-anti-rabbit IgG (whole molecule) (Sigma-Aldrich) or goat-anti-mouse IgG (H+L) (Kierkegaard & Perry Laboratories). Cells were analysed by epifluorescence microscopy using a Nikon Eclipse E600 microscope fitted with FITC-HYQ and

Texas Red HYQ filter blocks and a QImaging Micropublisher Digital CCD color camera. Digital images were processed using Adobe Photoshop CS and ImageJ version 1.33u (NIH) for Macintosh on an Apple Powermac G5.

Membrane and Protein Fractionations. Outer membrane vesicles (OMVs) were isolated and purified by treatment of *B. burgdorferi* cells with low pH, hypotonic citrate buffer followed by isopycnic sucrose gradient centrifugation as described (Skare et al., 1995). Briefly, early exponential phase *B. burgdorferi* cells were washed in 1x PBS containing 0.1% BSA, resuspended and incubated under vigorous shaking for 2 hrs in 25 mM citrate buffer, pH 3.2, containing 0.1% BSA. OMVs and protoplasmic cylinders (PCs) were fractionated by ultracentrifugation in a discontinuous gradient of 56, 42, and 25 % (wt/wt) sucrose in citrate buffer using a Beckman L8-80M centrifuge, SW28 rotor and 25x89 mm Ultra-Clear ultracentrifuge tubes. Fractions were washed and resuspended in 1x PBS containing 1mM PMSF.

Membrane proteins were extracted by detergent solubilization using a protocol modified from Brandt et al. (Brandt et al., 1990) and Nally et al. (Nally et al., 2001). Briefly, harvested *B. burgdorferi* cells were solubilized overnight in ice-cold PBS-Mg containing 2% (*v/v*) Triton X-114 with rotation at 4°C. Insoluble PC material was removed by centrifugation and the supernatant was phase-separated at 37°C for 15 min and centrifuged to obtain the aqueous periplasmic and detergent-soluble membrane fractions. Both the aqueous and detergent fractions were washed three times by addition of ice-cold Triton-X114 to the aqueous phase at 2% final

concentration, or ice-cold PBS+Mg to the detergent phase and phase-separated as above. Proteins were concentrated by acetone precipitation.

Radiolabeling of *Borrelia* Lipoproteins. Lipidation of recombinant proteins was confirmed by metabolic labeling of *B. burgdorferi* with [9,10(n)-^{3}H]palmitic acid (Amersham Biosciences) using a protocol modified from Brandt et al. (Brandt et al., 1990). Briefly, about $5x10^{7}$ early log-phase spirochaetes were washed once with PBS+Mg and resuspended in 1.0 ml fresh BSK-II containing 150 μCi tritiated palmitate and appropriate antibiotics. The cells were then incubated at 34˚C for 48 h, washed twice with PBS+Mg, and resuspended in SDS-PAGE sample buffer. Next, whole cell proteins were fractionated by SDS-PAGE. The gels were stained with Coomassie brilliant blue, destained and fixed with methanol and acetic acid, and immersed in Amplify fluorographic reagent (Amersham Biosciences) for 30 minutes. After drying under vacuum for 2h at 60°C, the gels were exposed overnight to x-ray film at -80°C.

Immunoprecipitation. Proteins were isolated from whole cell lysates by immunoprecipitation using protein-specific antibodies and Protein A Sepharose 4 Fast Flow beads (Amersham Biosciences) following the manufacturers' instructions. Briefly, $5x10^{9}$ spirochaetes were harvested and washed twice in ice-cold PBS-Mg. Cells were resuspended in 1 ml of ice-cold NP40 lysis buffer (150 mM NaCl, 1% IGEPAL CA-630, 50 mM Tris, pH 8.0, 1 mM PMSF) and lysed on ice for 15 min

with occasional gentle mixing. Cellular debris was pelleted by centrifugation for 10 min at 15,000 x *g* and 4˚C. 500 µl of the supernatant was incubated with 5 µl of polyclonal anti-mRFP1 for 1 h at 4˚C with over-end mixing. Next, 50 µl of washed protein A beads were added and the incubation continued for 1 h, after which the beads were washed 3 times with lysis buffer and once with 50 mM Tris, pH 8.0. Immunoprecipitated protein was released from the beads by boiling for 4 min in 50 µl of SDS-PAGE sample buffer, collected in 40 µl of supernatant and analysed by SDS-PAGE.

Chapter III: Single deletions in the unstructured N-terminus of *Borrelia burgdorferi* lipoprotein OspA reveal a sequential order of translocation through the outer membrane

Abstract

Borrelia burgdorferi surface lipoproteins are essential to the pathogenesis of Lyme disease, but the mechanisms responsible for their localization are only beginning to emerge. We have previously demonstrated the critical nature of the unstructured amino-terminal 'tether' domain of the mature lipoprotein for sorting a fluorescent reporter to the *Borrelia* cell surface. Here, we provide additional insights into the role of the lipoprotein tether by showing that deletion of four key amino acid residues within this tether results in the inefficient translocation of the major surface lipoprotein OspA across the *Borrelia* outer membrane. The carboxy-termini of these mutants are surface-exposed, but the proteins fail to be fully translocated. An expansive *in silico* analysis indicates that amino-terminal disorder is a feature common to lipoproteins of all bacterial phyla. This suggests an important role for lipoprotein tethers in proper localization within the *B. burgdorferi* cell envelope as well as the membranes of other distantly related bacteria.

Introduction

Bacterial lipoproteins are a ubiquitous subclass of membrane proteins characterized by a covalent N-terminal acyl modification (Hantke,1973). The addition of a fatty acid group to the peptide allows for the sequestration of the protein to a membrane compartment, consequently permitting these proteins to perform functions specific to the membrane leaflet into which they are anchored. These specialized proteins have well-documented roles in bacterial physiology and pathogenicity. For example, lipoproteins present in the periplasmic leaflet of the Gram-negative outer membrane (OM) help anchor the rigid peptidoglycan-based cell wall to the fluid bacterial membrane (Cascales,2002), and surface-localized *Bordetella pertussis* SphB1 is required for the maturation of its major antigen, filamentous hemagglutinin (Coutte,2003)). *Borrelia burgdorferi,* the bacterial agent of Lyme disease and the focus of this study, possesses numerous surface lipoproteins, many of which have been found to contribute to the pathogenesis of the organism (Fraser,1997;Haake,2000).

All lipoproteins are synthesized in the cytoplasm with a signal II peptide that contains a 'lipobox' lipidation motif ending in an absolutely conserved cysteine residue (von Heijne,1989). This signal sequence is removed following transport across the cytoplasmic membrane and the N-terminal cysteine becomes acylated, resulting in a mature lipoprotein attached to the periplasmic face of the cytoplasmic membrane (Sankaran,1994).

Among the various strategies employed by bacteria for transporting proteins between and across membranes (Economou,2006), lipoproteins appear to use a dedicated system for proper cellular localization. In *Escherichia coli*, a five-component lipoprotein transport machinery (termed the Lol system for Lipoprotein Outer Membrane Localization) has been analyzed in great detail (for reviews, see (Narita,2004) and (Tokuda,2004)). The *E. coli* Lol system sorts lipoproteins on the basis of the identity of the amino acid immediately adjacent to the fatty-acylated cysteine residue. An aspartic acid at this '+2' position results in inner membrane retention; otherwise, the lipoprotein is transferred to the periplasmic face of the outer membrane via a periplasmic chaperone. We previously ruled out a '+2-rule' for transport of *Borrelia* lipoproteins and suggested that *B. burgdorferi* employs a modified lipoprotein transport mechanism based on an incomplete set of Lol proteins (Schulze,2006).

We have already performed an initial characterization of the N-terminal tether for OspA, a major *B. burgdorferi* surface lipoprotein and target of a first-generation Lyme disease vaccine (Schaible,1990;Li,1997;Pal,2004). A fusion of the first 28 amino acids of OspA to a monomeric fluorescent protein (OspA28:mRFP1) resulted in the lipidation and successful transport of the reporter to the Borrelia cell surface (Schulze,2006). Truncation mutations of the N-terminus revealed that sequence information sufficient for surface localization of the reporter was present within the first five residues (lipoCys-17 through Val-21) of the mature OspA. Empirical mutagenesis of the entire lipoCys-17 through Asn28 tether failed to reveal a mutation

that hindered transport of the reporter to the surface. Negatively-charged residues in proximity to the N-terminal Cys appeared to function as inner membrane retention signals, but only in a particular context. This led to our initial conclusion that, in the absence of a retention signal, *Borrelia* lipoproteins are transported to the cell surface by a default mechanism.

In this study, we further examined the role of residues within the tether of the OspA lipoprotein from *B. burgdorferi*. Site-directed mutagenesis identified a region crucial for the transport of OspA across the outer membrane, and epitope-tagging of mutants revealed a potential sequential mode of transport across the membrane. Based on an extensive in silico analysis, we hypothesize that the N-terminal lipoprotein tethers of *Borrelia*, as well as those of other bacterial phyla, have evolved to retain a disordered conformation essential for proper lipoprotein localization and full biological functionality.

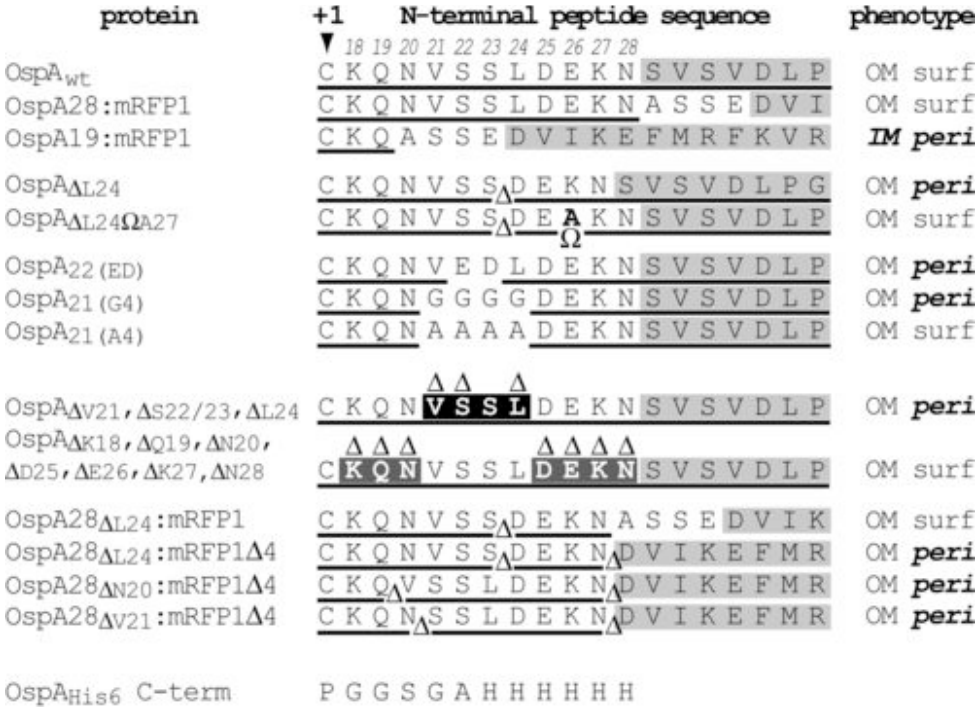
Results

OspA tether length itself does not dictate OM translocation. Our preliminary analysis of the OspA tether suggested a default export pathway of lipoproteins to the *Borrelia* cell surface (Schulze,2006). In the course of that study, we identified a single-residue tether deletion mutant of OspA, OspAΔL24. When expressed in *B. burgdorferi* strain KU-1038, this mutant was largely resistant to *in situ* proteolysis by proteinase K (Fig. 11; Fig. 12B, compare OspAwt to $OspA_{\Delta L24}$) and localized to the inner leaflet of the outer membrane.

We chose to further analyze the $OspA_{\Delta L24}$ mutant to determine the root cause for its mislocalization within the cell. Additional mutagenesis revealed protease sensitivity of $OspA_{\Delta L24}$ could be completely restored by the insertion of an alanine residue at an alternative location within the tether (Fig. 11; Fig. 12B, lanes 5 and 6). This suggested that the identity of the missing leucine residue itself was not the critical determinant of surface localization. Indeed, a previously described removal of Leu24 from a full-length OspA tether fusion to mRFP1 (Fig. 11; Fig 12A, lanes 5 and 6) had no detrimental effect on secretion to the cell surface. Because of different contexts of the Leu24 deletion in wild-type OspA and the OspA28:mRFP1 fusion, we surmised that other factors were contributing to the surface localization of OspA28:mRFP1.

mRFP1 is a monomeric derivative of dsRed (Campbell,2002). The first five amino-terminal residues of dsRed do not appear in its crystal structure (PDB: IG7K, (Yarbrough,2001)) due to the absence of electron density, indicative of potential flexibility within this region. Our original OspA:mRFP1 omitted only the N-terminal fMet of mRFP1. Based on the dsRed structure, we were therefore likely including an additional flexible linker between the OspA tether sequence and the structurally confined region of mRFP1. To determine whether this four residue linker of mRFP1 (Ala2-Ser3-Ser4-Glu5) contributed to the observed discrepancy between the localization of $OspA_{\Delta L24}$ and $OspA28_{\Delta L24}$:mRFP1, we removed it to yield the truncated reporter protein mRFPΔ4. A OspA28:mRFPΔ4 fusion was transported as effectively to the surface of *Borrelia* as OspA28:mRFP1, the original fusion to full-

Figure 11. Peptide sequences of OspA lipoprotein mutants. Deletions (Δ) and insertions (Ω) mutations with respect to the sequence of wild type (wt) OspA. Δ symbols within the sequence mark the deletion, and Δ symbols above the sequence indicate the deleted amino acid below. Gray shading indicates the structurally confined portion of the protein. Underlined sequence indicates the portion of the construct derived from wild type OspA. Mutant protein phenotypes are summarized by membrane (inner membrane, IM; outer membrane, OM), surface (surf) or periplasmic (peri) localization.

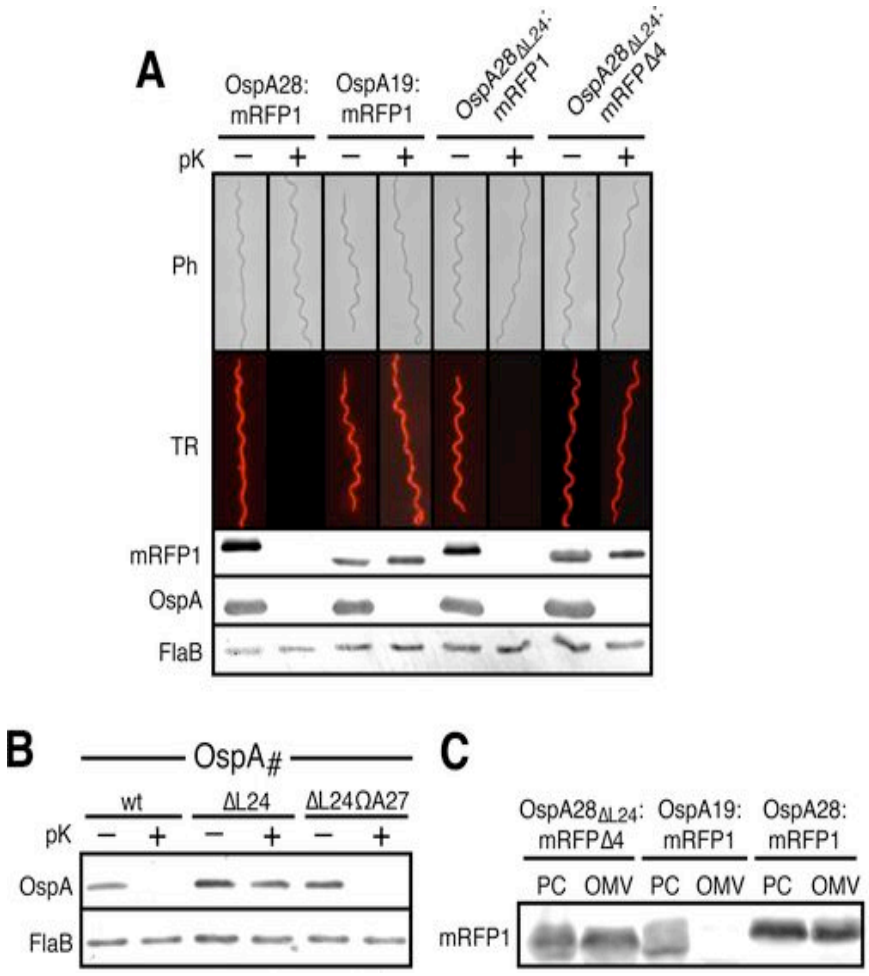

protein
+1 N-terminal peptide sequence
phenotype
18 19 20 21 22 23 24 25 26 27 28
OspA wt C K Q N V S S L D E K N S V S V D L P OM surf
OspA28:mRFP1 C K Q N V S S L D E K N A S S E D V I OM surf
OspA19:mRFP1 C K Q A S S E D V I K E F M R F K V R IM peri
OspA ΔL24 C K Q N V S S Δ D E K N S V S V D L P G OM peri
OspA ΔL24ΩA27 C K Q N V S S Δ D E A K N S V S V D L P OM surf
Ω
OspA22 (ED) C K Q N V E D L D E K N S V S V D L P OM peri
OspA21 (G4) C K Q N G G G G D E K N S V S V D L P OM peri
OspA21 (A4) C K Q N A A A A D E K N S V S V D L P OM surf
Δ Δ Δ
OspA ΔV21, ΔS22/23, ΔL24 C K Q N V S S L D E K N S V S V D L P OM peri
OspA ΔK18, ΔQ19, ΔN20, ΔD25, ΔE26, ΔK27, ΔN28
Δ Δ Δ Δ Δ Δ Δ
C K Q N V S S L D E K N S V S V D L P OM surf
OspA28 ΔL24:mRFP1 C K Q N V S S Δ D E K N A S S E D V I K OM surf
OspA28 ΔL24:mRFP1Δ4 C K Q N V S S Δ D E K N Δ D V I K E F M R OM peri
OspA28 ΔN20:mRFP1Δ4 C K Q Δ V S S L D E K N Δ D V I K E F M R OM peri
OspA28 ΔV21:mRFP1Δ4 C K Q N Δ S S L D E K N Δ D V I K E F M R OM peri
OspA His6 C-term P G G S G A H H H H H H

length mRFP1 (Fig. 12A). Removal of Leu24 from this new fusion resulted in a phenotype that behaved in all respects like the OspAΔL24 mutant: OspA28$_{\Delta L24}$:mRFPΔ4 was resistant to proteinase K digestion (compare lanes 7 and 8 of Fig. 12A and lanes 3 and 4 of Fig. 12B) and also localized to the outer membrane with the same efficiency as surface-exposed OspA28:mRFP1. The Leu24 deletion mutant thus represents a lipoprotein with a true defect in translocation across the outer membrane (Fig. 12C, compare lanes 5 and 6 to lanes 1 and 2).

To determine whether the deletion of any single residue within the tether of OspA resulted in a general defect in transport of lipoproteins across the outer membrane, we chose a second residue, Asn20, and removed it from the tether of both wild-type OspA and OspA28:mRFPΔ4, creating OspA$_{\Delta N20}$ and OspA28$_{\Delta N20}$:mRFPΔ4, respectively. In both instances, this mutation had no effect on the transport of the respective lipoproteins to the cell surface (see Fig. 13A, lanes 3 and 4; Fig. 13B, lanes 3 and 4).

Broadening our search, we next individually removed each of the remaining residues from the OspA tether to search for a phenotype similar to the Leu24 defect. Indeed, removal of Val21 and Ser at positions 22 or 23 had a sorting defect. As with Asn20, deletion of residues 18-19 and 25-28 had no impact on cell surface localization of OspA (Fig. 13A). Like OspA$_{\Delta L24}$, the OspA$_{\Delta V21}$ and OspA$_{\Delta S22/23}$ mutants localized to the OM with OspA$_{wt}$-like efficiency and were resistant to proteinase K treatment (Fig. 13D). However, the resistance to protease was approximately two-fold higher for the Val21 and Ser22/23 deletion mutants versus

Figure 12. Role for OspA Leu24 for OM translocation. (A) Epifluorescence micrographs of *B. burgdorferi* expressing various red fluorescent protein fusions before and after treatment with proteinase K (pK). Ph, phase contrast; TR, Texas Red filter. Supporting Western immunoblots for mRFP1, using surface-exposed OspA and periplasmic FlaB as controls are shown below. (B) Proteinase K accessibility of OspA tether mutants compared to $OspA_{wt}$. FlaB is used as a periplasmic, protease-resistant control. (C) Membrane fractionation immunoblots of $OspA_{\Delta L24}$:mRFPΔ4 compared to surface-localized OspA28:mRFP1 and IM-localized OspA19:mRFP1 controls (Schulze,2006) OM, outer membrane vesicle fraction; PC, protoplasmic cylinder fraction (also containing intact cells; ref. (Schulze,2006)).


A
OspA28: mRFP1
OspA19: mRFP1
OspA28ΔL24: mRFP1
OspA28ΔL24: mRFPΔ4
pK
Ph
TR
mRFP1
OspA
FlaB
B
OspA#
wt
ΔL24
ΔL24ΩA27
pK
OspA
FlaB
C
OspA28ΔL24: mRFPΔ4
OspA19: mRFP1
OspA28: mRFP1
PC
OMV
mRFP1

the Leu24 mutant (Fig. 13B). This indicates a clear hierarchy of importance for residues within the OspA tether for OM translocation.

A previous alanine scanning mutagenesis of the OspA28:mRFP1 fusion tether found no effect on surface localization (Schulze,2006). To extend these studies to full-length OspA, we changed the VSSL sequence examined above to AAAA. The resulting $OspA_{Ala(4)}$ mutant still successfully translocated to the cell exterior (Fig 14A, lanes 3 and 4). Changing the sequence to GGGG, however, resulted in a significant sorting defect: the resulting $OspA_{Gly(4)}$ mutant was as protease-resistant as $OspA_{\Delta V21}$ and $OspA_{\Delta S22}$ (Fig 14A, lanes 1 and 2) and was also found to localize to the OM (Fig. 14B, lanes 3 and 4). Changing both serine residues at positions 22 and 23 to Glu/Asp respectively (Fig. 14A, 14C), resulted in the same OM mislocalization phenotype. Since removal of only certain residues from within the tether resulted in a defect and both OspAGly(4) OspA22(ED) were also found to be mislocalized, we were able to rule out a singular role of tether length in the translocation process. Having identified the residues critical to OM translocation of OspA, we next sought to clarify their specific contribution to this process.

Epitope-tagging of mutants reveals a potential C-terminal-first mechanism for lipoprotein surface exposure. We engineered a hexa-histidine epitope tag with a Pro-Gly-Gly-Ser-Gly-Ala linker onto the C-terminal ends of OspA, $OspA_{\Delta S22}$, and $OspA_{\Delta L24}$. Western blotting using an antibody to OspA showed that addition of this C-terminal tag to wild-type OspA did not affect the surface-exposure of the protein

(Fig 14C, lanes 1 and 2). $OspA_{\Delta S22}$-His and $OspA_{\Delta L24}$-His, like their untagged counterparts, were largely protected from proteinase K treatment (Fig. 14C). Western blots using a monoclonal antibody against the C-terminal 6xHis tag, however, showed that the His tag for all three proteins was accessible to the protease. Accordingly, the apparent molecular weight of the OspA protein band decreased slightly following proteolytic treatment of the His-tagged constructs (Fig. 14C, lanes 3-6). This mobility shift was not observed when non-His tagged subsurface mutants of OspA were treated with proteinase K (see Fig 14A, lanes 1 and 2). The estimated 1-2 kDa decrease is comparable to the size increase seen upon adding the 1.3 kDa linker-His-tag peptide and suggests that only the extreme C-terminus is proteolytically cleaved. This could indicate that the C-terminal His tag is exposed on the surface of the cell while the remainder of the protein, i.e. the OspA portion, remains sequestered in the periplasm. If true, this would indicate that the subsurface mutant proteins may be locked in a transition state in their movement across the membrane and that translocation across the membrane originates at the C-terminal end of the protein. This leads to a model of lipoproteins threading C-terminus-first through a pore in the *Borrelia* OM (Fig. 15).

N-terminal disorder is a conserved feature of other bacterial lipoproteins. As a result of the great diversity of functions for bacterial lipoproteins, it is not surprising that there is little overall primary sequence conservation within these proteins outside of their lipobox. We performed a comparative analysis of the N-terminal sequence

Figure 13. Localization of single-residue deletion OspA tether mutants. (A) Protease accessibility assays for individual residue deletions from the OspA tether. (B) % proteinase K resistance of OspA mutants was calculated from Coomassie-stained gel densitometry of three independent *in situ* proteolysis assays. (C) Epifluorescence micrographs of *B. burgdorferi* B313 expressing various OspA tether-mRFPΔ4 fusions. (D) Membrane fractionation immunoblots of single-residue OspA deletions mutants compared to $OspA_{wt}$. OppAIV serves as IM control. Other labels for all panels are as in Fig. 2.

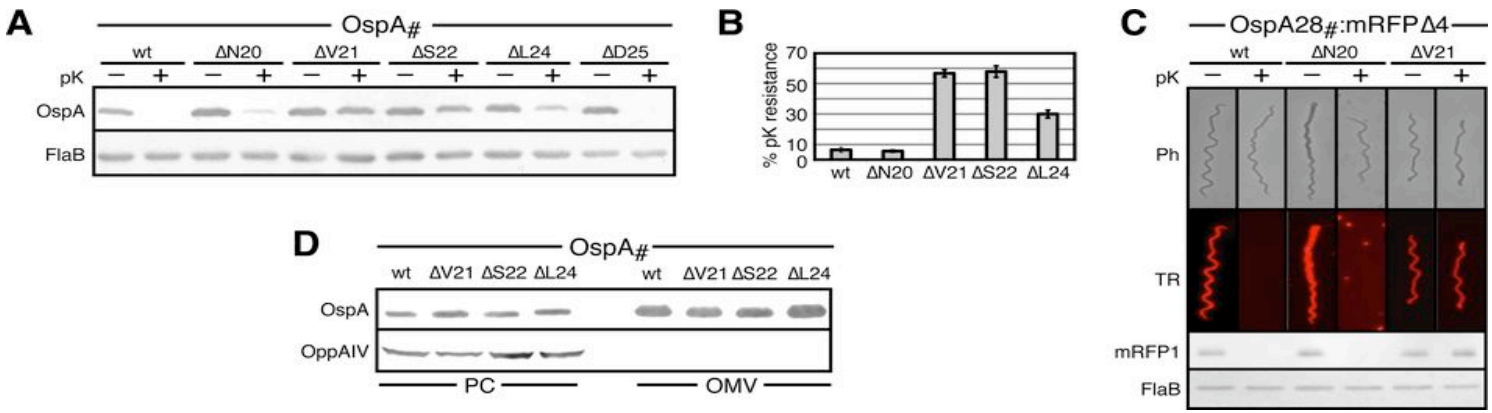

A
OspA#
wt
ΔN20
ΔV21
ΔS22
ΔL24
ΔD25
pK
OspA
FlaB
B
% pK resistance
70
50
30
10
0
wt
ΔN20
ΔV21
ΔS22
ΔL24
C
OspA28#:mRFPΔ4
wt
ΔN20
ΔV21
pK
Ph
TR
mRFP1
FlaB
D
OspA#
wt
ΔV21
ΔS22
ΔL24
OspA
OppAIV
PC
OMV

Figure 14. Localization of substitution OspA tether mutants and a C-terminal epitope tag. (A) Proteinase K accessibility assay immunoblots for Val-Ser-Ser-Leu OspA tetrapeptide substitution mutants. (B) Membrane fractionation of the VSSL mutants compared to wild-type OspA. (C) Epitope-tagging of subsurface mutants selective surface accessibility. Note the 1-2 kDa shift of $OspA_{\Delta S22}$-His6 and $OspA_{\Delta L24}$-His6 proteins upon *in situ* proteolytic treatment. Other labels for all panels are as in Fig. 2.

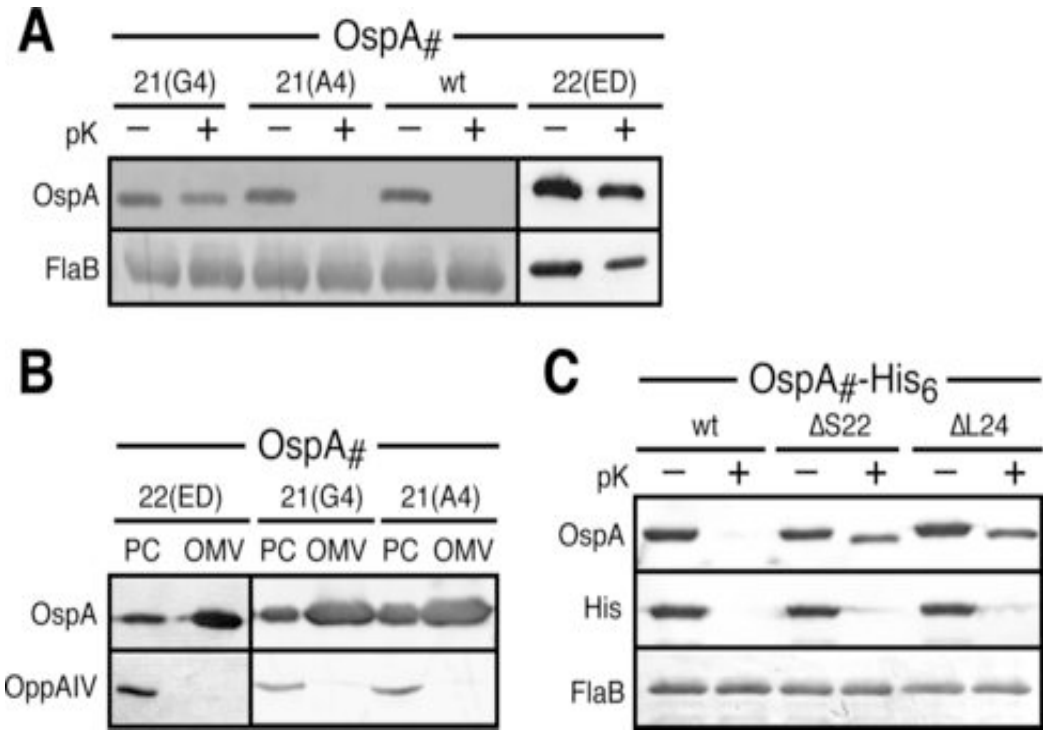
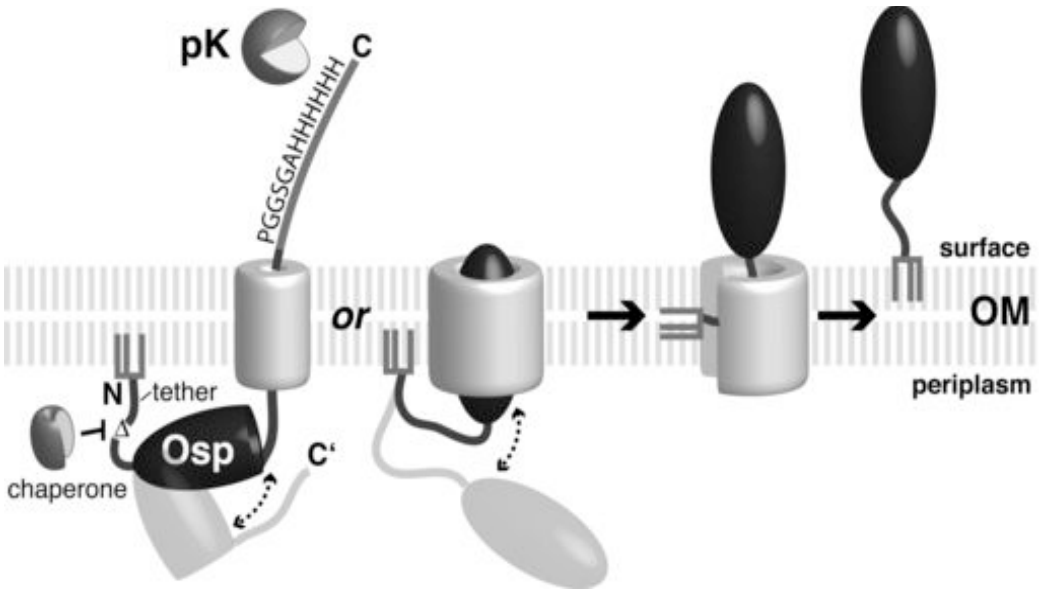

A
OspA#
21(G4)
21(A4)
wt
22(ED)
pK
– + – + – + – +
OspA
FlaB
B
OspA#
22(ED)
21(G4)
21(A4)
PC OMV PC OMV PC OMV
OspA
OppAIV
C
OspA#-His6
wt
ΔS22
ΔL24
pK
– + – + – +
OspA
His
FlaB

Figure 15. Model for *Borrelia* lipoprotein OM translocation. Current model for a 'tail-first' translocation of OspA (black with C-terminal epitope tag extension) through a hypothetical OM translocation core in either a partially unfolded or folded conformation. Certain tether deletion (Δ) mutants lead to an abortive translocation event, potentially due to a lack of interaction with a chaperone, releasing lipoprotein back to the periplasmic space. The N-terminal acyl group of wild type OspA ultimately flips to the external OM leaflet through a 'card-reader' mechanism. This likely requires a lateral opening of the proposed translocation/flippase channel.


pK
C
PGGSGAHHHHHH
or
surface
OM
periplasm
N
tether
Δ
Osp
C
chaperone

from 90 experimentally verified *E. coli* K12 lipoproteins (Tokuda,2007) and 127 known or predicted *B. burgdorferi* B31 lipoproteins (Setubal,2006). Examination of the first twenty residues of the mature lipoproteins for these two organisms revealed no readily identifiable sequence motifs from positions +2 to +20 (Tables 4-6;Fig. 16A). Aside from the universally-conserved N-terminal cysteine at position +1, most other positions had low levels of residue conservation.

We next examined representative lipoproteins from all bacterial phyla and found that nearly all lipoproteins whose structures have been determined by X-ray crystallography share the common feature of containing an N-terminal region devoid of electron density (Table 3). Since structures have been solved for only a small percentage of *E. coli* and *B. burgdorferi* lipoproteins, we used the VSL2B algorithm (Peng,2006) to predict the likelihood of N-terminal disorder for all known and predicted lipoproteins for these two organisms. The calculated mean probability of disorder was plotted as a function of residue position from the N-terminal cysteine. This analysis revealed a remarkable gradient for both *E. coli* and *B. burgdorferi* lipoproteins, with the calculated probability higher for residues proximal to the amino terminal cysteine compared to those further along the polypeptide chain (Fig. 16B). A comparison of *E. coli* lipoproteins to non-lipidated cytoplasmic and periplasmic proteins showed some degree of predicted amino terminal disorder for all three classes. However, the unstructured region of lipoproteins was predicted to be about four times as long (~23 vs. ~6 residues; data not shown). As the VSL2B algorithm uses a probability of 0.5 to differentiate a predicted “ordered” residue from

Table 3. Examples of N-terminal Unstructured Regions of Bacterial Lipoproteins

Organism	Protein	N-terminal Sequence of Mature Lipoprotein	Length†	Reference	PDB-ID
Escherichia coli	LolB	CSVTTPKGPGKSPD	14	{Takeda,2003}	1IWN
Escherichia coli	AcrA	CDDKQAQQGGQQMPAVGVVTVKTEPLQITT	30	{Mikolosko,2006}	2F1M
Shigella flexneri	MxiM	CALKSSSNSE	9	{Lario,2005}	1Y9L
Pseudomonas aeruginosa	MexA	CGKSEAPPPAQTPEVGIVTLEAQTVTLN	28	{Higgins,2004}	1T5E
Neisseria meningitidis	PilP	CSQGSEDLNEWMAQTRREAKAEIIPFQAPTLPVAPVYSPP QLTGPNAFDFRRMETDKKGENAPDTKRIK	65	{Golovanov,2006}	2IVW
Borrelia burgdorferi	OspA	CKQNVSSLDEKN	12	{Li,1997}	1OSP
Borrelia burgdorferi	OspC	CNNSGKDGNTSANSADESVKGP	22	{Kumaran,2001}	1GGQ
Borrelia burgdorferi	BbCRASP-1	CAPFSKIDPKANANTKPKKITNPGENTQNFEDKSGDLSAS DEKIME	46	{Cordes,2005}	1W33
Borrelia turicatae	Vsp1	CNNSGTSPKDGQAAKSDGTVI	21	{Lawson,2006}	2GA0
Leptosira interrogans	Lp49	CKSGDFSLLSSPINREKNG	19	{Giuseppe,2008}	3BWS
Treponema pallidum	PnrA	CSKSDRPQMGNAGGAEGGDF	20	{Deka,2006}	2FQW
Mycobacterium tuberculosis	LppX	CSSPKPDAEEQGVPVSPTA	19	{Sulzenbacher,2006}	2BYO
Streptococcus pneumoniae	PsaA	CASGKKDAASGQK	13	{Lawrence,1998}	1PSZ

† Length is defined as the number of N-terminal residues devoid of electron density in the associated crystal structure plus those up to but not part of the first secondary structural element (i.e. α-helix, β-sheet).

Table 4. *Escherichia coli* Lipoprotein N-termini

Lipoprotein	GeneID	Sequence	Lipoprotein	GeneID	Sequence	Lipoprotein	GeneID	Sequence
AcrA	1786668	CDDKQAQQGGQQMPAVGVVT	Slp	90111603	CSSIPQNIKGNNQPDIQKSF	YehR	90111391	CGDKEESKKFSANLNGTEIA
AcrE	16131153	CNDKGEEKAHVGEPQVTVHI	SlyB	49176129	CVNNDTLSGDVYTASEAKQV	YfeY	16130357	CSTMSSVNWSAANPWNWFGS
ApbE	16130151	CDQKPQPAKTHATEVTVLEG	Spr	16130113	CSANNTAKNMHPETRAVGSE	YfgH	16130430	CQSNADDHAADVYQTDQLNT
Blc	16131974	CSSPTPPRGVTVVNNFDAKR	VacJ	16130279	CASSGTDQQGRSDPLEGFNR	YfgL	16130437	CSLFNSEEDVVKMSPLPTVE
CsgG	16129000	CLTAPPKEAARPTLMPRAQS	Wza	16130002	CTVLPGSNMSTMGKDVIKQQ	YfhG	16130480	CVQNHNKPAIDTPAEEKIPV
CyoA	16128417	CNSALLDPKGQIGLEQRSLI	YaeC	16128190	CGQDEKDPNHIKVGVIVGAE	YfiB	16130526	CQSPQGKFTPEQVAAMQSYG
DcrB	83305676	CDDKDTNATAQGSVAESNAT	YaeF	90111098	CTVDISQPDPSATAVDAEAK	YfiL	90111465	CQIDPYTHAPTLTSTDWYDV
EcnA	48994998	CNTARGFGEDIKHLGNSISR	YafT	16128203	CASESSIDEKKKKAQVTQSN	YfiO	16130516	CSGSKEEVPDNPPNEIYATA
EcnB	49176463	CNTTRGVGEDISDGGNAISG	YafY	145698221	CDDRSDDLKAISKFKDLTPP	YfjS	90111472	CDDRSDDLKAISKFKDLTPP
FlgH	16129042	CAWIPSTPLVQGATSAQPVP	YaiW	16128363	CSSQAPQPLKKGEKAIDVAS	YgdI	90111491	CSGSNYVMHTNDGRTIVSDG
HslJ	16129340	CVSNDKIAVTPEQLQHHRFV	YajG	90111134	CAKPPTTIEVSPTITLPQQD	YgdR	16130737	CSSDYVMATKDGRMILTDGK
LolB	16129172	CSVTTPKGPGKSPDSPQWRQ	YajI	90111129	CVQQSEVRQMKHSVSTLNQE	YgeR	90111503	CSGSKSSDTGTYSGSVYTVK
Lpp	16129633	CSSNAKIDQLSSDVQTLNAK	YbaY	49176025	CADKSADIQTPAPAANTSIS	YggG	90111514	CQNMDSNGLLSSGAEAFQAY
MltA	16130720	CSSKPTDRGQQYKDGKFTQP	YbcU	16128540	CAQQTFTVQNKQTAVAPKET	YghG	16130871	CASHNENASLLAKKQAQNIS
MltB	16130608	CSSKPKPTETDTTTGTPSGG	YbfN	16128658	CAQSTAPQEDSRLKEAYSAC	YhfL	16131247	CTGHIENRDKNCSYDYLLHP
MltC	90111520	CSTTKKGDTYNEAWVKDTNG	YbfP	16128665	CAQPEQSSLAGDWLLTPKDK	YhiU	16131385	CDDKSAENAAAMTPEVGVVT
MltD	16128198	CQSTGNVQQHAQSLSAAGQG	YbhC	16128740	CSSTPPDQRPSDQTAPGTSS	YiaD	49176370	CTTNPYTGEREAGKSAIGAG
MltE	90111231	CSSKHDYTNPPWNAKVPVQR	YbjP	16128833	CTTVTPAYKDNGTRSGPCVE	YidQ	90111638	CSSVMSHTGGKEGTYPGTRA
NlpA	16131531	CDQSSSDAKHIKVGVINGAE	YbjR	16128835	CAGEKGIVEKEGYQLDTRRQ	YiiG	16131736	CDDKKAETETLPPANSQPAA
NlpB	90111442	CSSDSRYKRQVSGDEAYLEA	YcaL	90111186	CQNTHGIDTNMAISSGLNAY	YjbF	90111676	CSATTKELGNSLWDSLFGTP
NlpC	16129664	CSHHKAPPPNARLSDSITVI	YccZ	16128949	CTLVPGQNLSTSNKDVIELP	YjeI	90111692	CSSSNELSAAGQSVRIVDEQ
NlpD	16130649	CSDTSNPPAPVSSVNGNAPA	YcdR	16128987	CISQSRTSFIPPQDRESLLA	YjfO	90111701	CSALQGTPQPAPPVTDHPQE
NlpE	16128185	CNNRAEVDTLSPAQAAELKP	YceB	16129026	CNQLTQYTITEQEINQSLAK	YlcB	16128555	CSLAPDYQRPAMPVPQQFSL
NlpI	16131055	CSNTSWRKSEVLAVPLQPTL	YcfM	16129068	CVGQREPAPVEEVKPAPEQP	YmcC	16128952	CTHSQQSMVDTFRASLFDNQ
OsmB	16129244	CSNWSKRDRNTAIGAGAGAL	YcjN	16129271	CKEENKTNVSIEFMHSSVEQ	YnbE	16129343	CTPRIEVAAPKEPITINMNV
OsmE	16129693	CTAYDRTKDQFVQPVVKDVK	YdhY	16129630	CKQEDIDSGTVGLINTPKGV	YnfC	90111299	CDRTEVTLSFTPEMASFSNE
Pal	16128716	CSSNKNASNDGSEGMLGAGT	YeaY	16129760	CVTVPDAIKGSSPTPQQDLV	YoaF	16129747	CSTPSQPEAPKPPQIGMANP
RcsF	16128189	CSMLSRSPVEPVQSTAPQPK	YecR	16129854	CTVTRQAHVSEVDAATGIVR	YqhH	16130912	CVNEQKVNQLASNVQTLNAK
RlpA	16128616	CTSDDGQQQTVSVPQPAVCN	YedD	16129875	CAEVENYNNVVKTPAPDWLA	Yrak	16131037	CAYASSGGCGADSTSGATNY
LptE(RlpB)	16128624	CGWHLRDTTQVPSTMKVMIL	YegR	90111383	CAVHNDETSIGKFGLAYKSN	YraP	16131042	CVAAAVVGTAAVGTKAATDP

Table 5. ***Borrelia burgdorferi*** **Lipoprotein N-termini**

Lipoprotein	GeneID	Sequence	Lipoprotein	GeneID	Sequence	Lipoprotein	GeneID	Sequence	Lipoprotein	GeneID	Sequence
BB_Q46	6382440	CKIWIVG	BB_K07	2690145	CKWHVDNPIDEATAESKSAL	BB_A04	2690227	CNLDDNSKMERKGSNKLIRE	BB_R28	6382188	CNSNDTNNSQTKSRQKRDLT
BB_A62	2690240	CETTRISDEMENTSDEDSKV	BB_K12	2690150	CKWYVDNTIDEATVESKSAL	BB_B09	2689911	CKSIPNGNFNLHDTNHKLGK	BB_O28	6382283	CNSNDTNTKQTKSRQKRDLT
BB_A25	2690243	CSIGLVERTNAALESSSKDL	BB_N39	6382367	CKNFATGKDIKQNSEGKIKG	BB_A64	2690254	CSLEVKDSNESKKHKKEKRK	BB_L28	6382328	CNSNDNDTLKNNAQQTKSRR
BB_0365	2688271	CTSKDSSNEYVEEQEAENSS	BB_S41	6382143	CKIDASSEDLKQNVKEKVEG	BB_0144	2688024	CDEKKSSKNLKSVKIGYVNW	BB_0823	2688767	CNKNNKIPLIQKLDLPKSSI
BB_A15	2690259	CKQNVSSLDEKNSVSVDLPG	BB_R40	6382197	CKNNTLSLYDEQSIG	BB_A07	2690229	CKEFNYSDLRRRPSKVLNAS	BB_0652	2688574	CLLIFPTLKWYFLMSVEDKK
BB_B19	2689901	CNNSGKDGNTSANSADESVK	BB_M38	6382234	CKNYASGEDVKKSLEQDLKG	BB_0806	2688754	CLTTDRSIQDSHISDIVEKK	BB_A36	2690262	CKQFGDVKSLTEIDSGNGIP
BB_B16	2689891	CVNESNRNKLVFKLNIGSEP	BB_K48	2690138	CKLYKKITYNADQVIDKLKS	BB_K52	2690128	CFLTPPKSSKINSIKTEVLD	BB_B25	2689907	CKILNIAEDLEKNFEKIERA
BB_B14	2689892	CTSLNVEHDQFGKTFRIYQS	BB_0385	2688297	CSSSDDGKSEAKTVSLIVDG	BB_0758	2688693	CNVVDTDFSVLEFKVANFNL	BB_N28	6382358	CNSNDTNTSQTKSRQKRDLT
BB_A24	2690249	CGLTGATKIRLERSAKDITD	BB_F20	2689993	CDLSSNNAENKMDDIFNLEK	BB_P28	6382099	CNSNDNDTLKNNAQQTKSRG	BB_B08	2689912	CNISVSSIFIRPLDEVIKSE
BB_J36	2690202	CKFFGNKSASKEKEETSFSD	BB_G25	2690009	CESIPSLPQKPTLTNKEDIE	BB_M28	6382224	CNSNDNDTLKNNAQQTKSRG	BB_0536	2688453	CVSNELKLDQSLVKGKLVNG
BB_A57	2690269	CNANMDTNDKNKALNEYKLK	BB_0298	2688168	CGNESKEKSNLGLRLRELEI	BB_Q35	6382414	CNSNDNDTLKNNAQQTKSRK	BB_A66	2690255	CTIDANLNEDYKNKVKGILN
BB_0323	2688219	CKTPPESRESKNAKIAQPDN	BB_K19	2690164	CNLFSKDSRSRQKYNFKVPA	BB_I14	2690098	CNFDNDAEAATKKHADKIKN	BB_J47	2690221	CATFVWLIGIFYSNNFKEER
BB_0328	2688228	CISNAKKEKIVFRVSNLSEP	BB_0155	2688044	CNTKTLNELGEEQFKIPFGT	BB_I29	2690116	CTANHEAEAKIKKHVDKTKN	BB_0224	2688117	CRVKGIVIKNGNCIKAKGIS
BB_0330	2688226	CNNNSEKEKLAFKVYIGGAP	BB_0464	2688383	CTFDYDEYSSRSDVAKKFPS	BB_A73	2690256	CSFYSKSNNTEAISELQSSP	BB_0475	2688417	CMKTSTIKSKENAKEITYLI
BB_A16	2690242	CAQKGAESIGSQKENDLNLE	BB_Q05	6382396	CNSDFSTNQEDIKYPSDKEK	BB_0840	2688783	CALIADNKSKNLSTSEIILT	BB_0460	2688384	CSKSVSSKVNSEFEIKTKNI
BB_0141	2688026	CVGDNKLDDKNIDKEKESSY	BB_A34	2690261	CSAMSKPKDDIVFGVGIGNE	BB_0542	2688469	CSSIFKEYQNISGEYYKLAK	BB_A65	2690283	CDLNNKDNKDKVASFTETKY
BB_O39	6382266	CKNYASGENLKNSEQNLESS	BB_J09	2690176	CVHDKQELSSKSNLNNQKGY	BB_0383	2688281	CSGKGSLGSEIPKVSLIIDG	BB_0213	2688119	CDINYPEIKELDYKINYYFT
BB_0664	2688594	CNHKNIQYDKRIKKFLDKNK	BB_Q03	6382395	CYLPDNQEQAVQTFFENSES	BB_P39	6382083	CKNYAIKDLEQNAKGKIKGF	BB_0628	2688555	CSSNVEIELNDDISGIVSIF
BB_0398	2688310	CNQKQSEIQNLTHLLKSSNK	BB_K53	2690129	CYLPDNQEQAVQTFFENSES	BB_L40	6382312	CKNYAIKDLEQNAKGKIKGF	BB_0171	2688069	CSNVKSMSLMAIGNYEYVRG
BB_0382	2688282	CFSRNGIESSSKKIKISMLV	BB_I42	2690089	CYLPDNQEQAVQTFFENSES	BB_A60	2690238	CDLSINKEQKTKEKTSEKQE	BB_0456	2688369	CRSESRLAENVLIEFFDSIK
BB_I28	2690115	CSTDFNTDQKGIKYPPTEKS	BB_0384	2688280	CFKSNKKSIKSDKVVVGVLA	BB_H06	2690054	CDVSRLNQRNINELKIFVEK	BB_A32	2690292	CSSFNKSSNKSLLAKNKQKA
BB_I16	2690100	CRPDFNIDQKDIKYPPTEKS	BB_A05	2690228	CKGKSLEEDLKSTTSNNKQN	BB_0844	2688797	CNTSDPNELTRKKMQDKNVK	BB_0215	2688115	CKNQDNEKIVSIGGSTTVSP
BB_0028	2687919	CSSESIFSQLGNLQKIKHEY	BB_0689	2688622	CNLSTMHKIDTKEDMKILYS	BB_E31	2689955	CNPDSNTNQNNSKKGLLKIE	BB_0227	2688132	CTKKLTKINFKDISLNSNKM
BB_A33	2690296	CYLNDFSGMKENNCNKYDLS	BB_A59	2690237	CSAIGRGILIDSILNNVHKE	BB_J01	2690206	CNPDSNTNQNNSKKELKTGR	BB_F01	2689987	CKFDSLNLSTKSVDDKNNSI
BB_P38	6382082	CKIHTSYDEQSNGEVKVKKI	BB_A69	2690286	CAPFNKINPKANENTKLKKN	BB_I34	2690120	CYPVASNKIELKPKTETSLN	BB_E04	2689962	CAPIGKVNTKPNSDTNPENN
BB_L39	6382311	CKIHTSYDEQSNGEVKVKKI	BB_I36	2690092	CAPFGNVNPNKLKNPITSKN	BB_H01	2690046	CNLSNSDQNNPLNMSNKEKI	BB_A03	2690226	CKNKSNDAEPNNDLDEKSQA
BB_N38	6382366	CKIHTSYDEQSSGEINHTLY	BB_I38	2690105	CAPFGNVNPNKLKNPITSKN	BB_Q89	6382465	CNLSNSDQNNPLNMSNKEKI	BB_K01	2690139	CNLDSKLSSNKEQKNNNNVK
BB_H18	2690078	CDWGTIKDKSTEISKLLRTD	BB_J41	2690183	CAPFGNVNPNEPKNPTTSKS	BB_K32	2690126	CDLFIRYEMKEESPGLFDKG	BB_H37	2690074	CNLNSKLSGNKEEQKNNNDI
BB_0832	2688779	CASLPYTPPKQNLNYLMELL	BB_I39	2690106	CAPFGNVNPNEPKNPTTSKS	BB_J08	2690188	CNLDSKLPNKEQKNNNDIKE	BB_G01	2690030	CNLDSKLSSNKEQKNNNNVK
BB_H32	2690052	CNPDFNTNKKRTLSKGIISN	BB_0158	2688051	CAFFKKPQSVHQDSNTGKPI	BB_0193	2688084	CKESSIIEKQFNYAIIFSDA	BB_A72	2690289	CAVNPIGPKVKSRTDIKESN
BB_J34	2690200	CKFYGSDDTNKKNTSLNGDT	BB_B27	2689905	CSTISLVKIPEKDKINLTVL	BB_P27	6382098	CKAYVEEKKEIDSLMEDVLA	BB_0639	2688565	CTNKDTITLNVFNWAEYIDK
BB_D10	2689938	CQTYQIAYDRFSQVLDSQYD	BB_Q47	6382424	CKIDATGKDATGKDATGKDA	BB_M27	6382223	CKAYVEEKKEIDSLMEDVLA	BB_A14	2690258	CSTIASLPEEPSSPQESTLK
BB_K50	2690127	CKLYEKLTNKSQQALAKAFV	BB_R42	6382172	CKNDVTSKDLEGAVKDLESS	BB_A68	2690285	CAPFSKIDPKANANTKPKKI	BB_0352	2688261	CSTNKNIVVLTDNKTIPFYI
BB_C10	2689921	CELFIIKRRATITETTTIEK	BB_E08	2689971	CNNYANDKGLKRVKEYLEKE	BB_0329	2688227	CNNKERKEGVSFKISLGAEP	BB_K47	2690174	CSGFLSKKSIEQFALALKDH
BB_O40	6382267	CKNYATGKDIKQNAKGKIKG	BB_0324	2688232	CYTINLEKLTKETPYGVYLR	BB_S30	6382132	CNSNDNDTLKNNAQQTKRRG	BB_K49	2690161	CSGFLSKKSIEQFALALKDH

Colors Indicate Status (Setubal, 2006) **True Negative** **True Positive** **False Negative**

Table 6. Residue Frequency by Position and Range

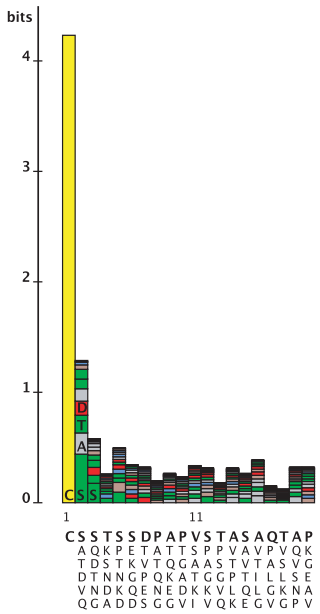
***Escherichia coli* K12**

Residue	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10	+11	+12	+13	+14	+15	+16	+17	+18	+19	+20	Frequency	+20	+15	+10	+5
A	0	13	5	6	5	6	5	8	10	9	9	9	4	12	11	14	8	6	14	8	A	162	112	67	29
C	90	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	1	C	5	2	1	0
D	0	9	10	6	7	7	12	7	4	8	4	4	4	5	5	7	4	5	4	1	D	113	92	70	32
E	0	0	2	5	5	10	7	3	9	2	6	6	6	1	7	5	1	4	5	8	E	92	69	43	12
F	0	0	0	1	0	1	1	1	1	2	1	1	2	2	0	1	5	3	1	1	F	24	13	7	1
G	0	3	6	0	3	9	2	7	7	9	4	8	8	7	4	7	7	7	5	8	G	111	77	46	12
H	0	0	2	5	3	0	1	3	1	4	2	1	1	0	1	1	2	0	2	0	H	29	24	19	10
I	0	1	1	2	3	1	5	4	1	3	7	1	2	4	2	8	4	4	4	3	I	60	37	21	7
K	0	2	1	11	7	9	4	6	9	5	8	7	5	7	4	3	2	7	2	9	K	108	85	54	21
L	0	1	3	4	2	1	2	4	5	5	2	3	3	7	4	7	7	7	5	4	L	76	46	27	10
M	0	0	1	2	1	0	1	2	1	2	1	3	2	3	3	1	1	2	0	0	M	26	22	10	4
N	0	6	7	8	7	5	5	7	5	5	6	1	5	2	6	4	3	6	8	3	N	99	75	55	28
P	0	0	1	5	14	6	8	10	4	10	5	11	10	9	5	6	10	4	5	11	P	134	98	58	20
Q	0	6	10	5	5	7	5	7	9	4	5	6	6	4	7	4	10	4	10	6	Q	120	86	58	26
R	0	0	1	4	4	5	2	4	4	1	2	2	2	2	3	0	2	2	3	5	R	48	36	25	9
S	0	30	23	8	16	10	6	6	4	5	10	14	9	5	12	4	5	7	8	6	S	188	158	108	77
T	0	11	9	11	7	6	10	7	9	9	8	2	11	9	7	8	6	11	3	6	T	150	116	79	38
V	0	8	5	5	0	3	9	3	5	6	10	6	6	9	7	9	6	7	9	7	V	120	82	44	18
W	0	0	2	1	0	1	0	0	1	0	0	1	2	1	1	0	2	2	0	0	W	14	10	5	3
Y	0	0	1	1	1	3	5	1	0	1	0	3	2	1	1	1	5	1	1	3	Y	31	20	13	3

***Borrelia burgdorferi* B31**

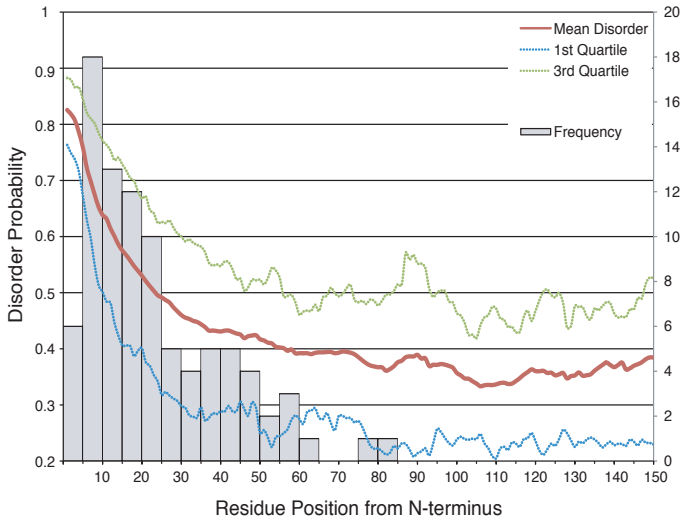
Residue	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10	+11	+12	+13	+14	+15	+16	+17	+18	+19	+20	Frequency	+20	+15	+10	+5
A	0	12	6	0	11	2	4	3	3	6	6	3	13	5	4	5	0	2	5	9	A	99	78	47	29
C	127	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	C	2	2	0	0
D	0	7	0	16	19	6	15	13	15	7	4	8	6	8	5	8	9	7	6	5	D	164	129	98	42
E	0	3	3	5	3	6	11	19	10	11	13	13	5	10	12	11	9	13	14	8	E	179	124	71	14
F	0	3	5	16	6	1	1	1	2	1	3	5	3	5	6	4	3	4	2	3	F	74	58	36	30
G	0	2	2	1	13	1	10	2	4	2	3	3	7	2	7	7	6	5	6	12	G	95	59	37	18
H	0	0	2	4	1	0	1	0	0	0	1	2	1	3	0	1	1	2	0	0	H	19	15	8	7
I	0	1	9	9	3	12	10	8	8	7	7	8	7	5	9	14	8	7	6	13	I	151	103	67	22
K	0	33	5	11	6	21	12	20	14	24	24	17	23	17	15	21	28	17	27	19	K	354	242	146	55
L	0	2	22	2	1	8	9	6	15	12	6	7	9	10	6	9	12	8	16	5	L	165	115	77	27
M	0	1	0	1	1	1	1	0	3	2	0	1	0	4	3	0	1	1	0	1	M	21	18	10	3
N	0	33	17	23	14	26	10	16	9	23	10	25	11	9	14	16	12	9	8	10	N	295	240	171	87
P	0	0	11	4	6	4	2	6	7	2	5	3	1	3	7	3	4	1	3	6	P	78	61	42	21
Q	0	1	4	0	2	1	4	4	13	8	4	11	6	8	11	1	2	2	2	0	Q	84	77	37	7
R	0	3	0	1	2	5	1	2	3	3	7	2	3	4	0	1	3	2	9	4	R	55	36	20	6
S	0	14	22	12	20	19	15	12	14	6	15	12	14	13	11	3	6	24	11	15	S	258	199	134	68
T	0	3	12	5	7	10	9	10	2	4	10	3	10	5	5	14	10	6	2	6	T	133	95	62	27
V	0	4	4	2	11	1	8	1	2	6	7	2	4	10	6	5	6	12	5	3	V	99	68	39	21
W	0	0	3	1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	W	7	6	6	4
Y	0	5	0	13	1	2	4	3	2	1	1	1	2	4	5	2	5	3	3	5	Y	62	44	31	19

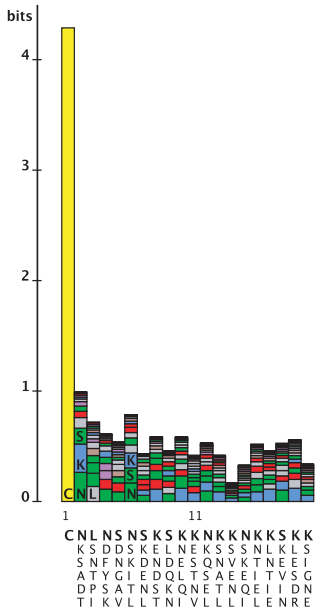
Figure 16. Sequence complexity and prediction of N-terminal disorder of *E. coli* and *B. burgdorferi* lipoprotein tethers. *(A)* A LogoBar (Perez-Bercoff,2006) representation of the N-terminal sequence of known or predicted mature lipoproteins for *E. coli* and *B. burgdorferi* (Tokuda,2007,Setubal,2006) illustrates the complexity of the tether. The height of each column is proportional to the lack of complexity at a given position. The columns are stacked from the bottom starting with the most frequently occurring residue at that position and continuing upward. Below each column are the six most frequently occurring residues at each position, in order of frequency from top (bold) to bottom. Colors represent residues with similar characteristics. *(B)* The VSL2B algorithm (ref. (Peng,2006); http://www.ist.temple.edu/disprot/predictorVSL2.php) for disorder prediction was used to generate a probability of disorder for each residue in each of 90 *E. coli* K12 lipoproteins (Tokuda,2007) and 127 known or predicted *B. burgdorferi* lipoproteins. The red line plots the average predicted disorder (left y-axis) as a function of position from the N-terminal lipidated cysteine (x-axis). In addition to the mean disorder, the first and third quartiles are listed to illustrate the dispersion of the probability values. Columns in the background show the number of lipoproteins (right y-axis) predicted to contain a tether of a length within the range indicated on the x-axis (e.g. there are five *E. coli* lipoproteins predicted to have a tether of between 40 and 45 residues in length).



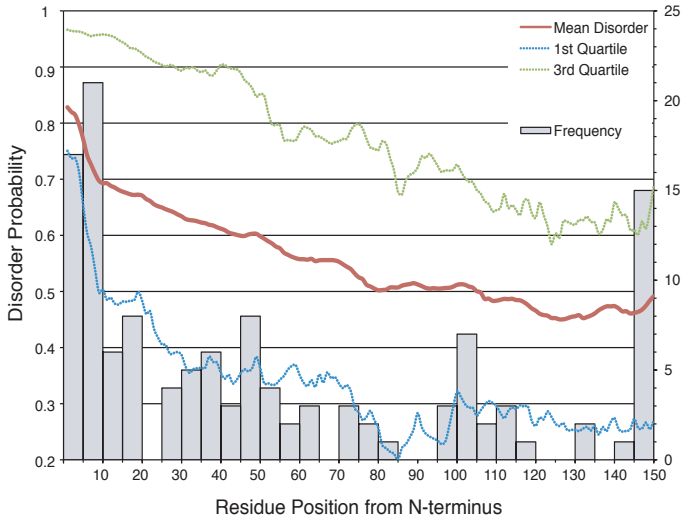
B

Escherichia coli (n=90)





Borrelia burgdorferi (n=127)



"disordered," we used the same cutoff to estimate the tether lengths for lipoproteins of *E. coli* and *B. burgdorferi*. The average tether length for *E. coli* lipoproteins was found to be approximately 24 residues in length. The average tether length for *B. burgdorferi* lipoproteins was predicted to be much longer, at 53 residues, with numerous lipoproteins predicted to have tethers of 150 residues or greater.

Discussion

In this study, we characterized and assigned a function to the OspA tether, the flexible unstructured region at the N-terminus of the OspA lipoprotein of *Borrelia burgdorferi*. We showed that a four-residue Val21-Ser22-Ser23-Leu24 sequence within the OspA tether contains information for optimal transport of the entire protein across the OM. The individual removal of any of these four residues severely impacted OspA OM translocation. Further analysis of C-terminally His-tagged mutants revealed a likely mechanism whereby OspA may cross the OM. We also presented preliminary evidence that tether domains may be a universal property of eubacterial lipoproteins.

There is little amino acid sequence similarity between mature lipoproteins, which likely reflects the enormous functional diversity of their globular moieties. Our work in this paper, together with structural determination and secondary structural predictions of numerous lipoproteins, suggests that the extreme amino-terminus is often disordered (Table 3). Examination of lipoproteins from distantly-related bacteria reveals that the N-terminal primary sequence of many mature

lipoproteins are well-populated by certain residues and sequences unlikely to be conducive to the formation of secondary structure, particularly Gly, Gln, Ser, Pro, Asp, Asn, Glu, and Lys (Romero,2001;Madan Babu,2002;Babu,2006;Peng,2006). Lys, Asn, Ser Asp and Glu, found to be especially common in regions of short disorder (Peng,2006), are the five most common residues within the first fifteen positions of the 127 known/predicted *B. burgdorferi* lipoproteins.

The lipoprotein tether acts as a physical link between the lipidated, membrane-bound cysteine and the globular portion of the protein. While the tethers themselves do not appear to be conserved at the level of their primary sequences, they do appear to be a common property of lipoproteins from all bacterial phyla. Because lipoproteins have long been recognized as both virulence determinants and potent immunostimulatory molecules, much interest surrounds their accurate identification in the genomes of known and emerging pathogens (Aliprantis,1999;Brightbill,1999;Hirschfeld,1999;Haake,2000;Sutcliffe,2002;Juncker, 2003). Currently, prediction of peptide lipidation is dependent only on the presence of a calculated lipobox consensus sequence (Nakai,1999;Juncker,2003;Babu,2006;Setubal,2006). The prediction of regions of disorder immediately adjacent to a signal-II peptidase recognition site may aid these lipoprotein prediction algorithms in the accurate identification of true lipoproteins.

The presence of N-terminal unstructured regions is not unique to those lipoproteins found in *Borrelia.* Nearly all crystal structures of lipoproteins deposited in the Protein Data Bank are missing large segments of N-terminal sequence.

Lipoproteins that have not been crystallized show other indications of disorder: SphB1 of *Bordetella pertussis* is an autotransported surface-anchored lipoprotein that has an unusual N-terminal sequence containing a 14-glycine stretch followed by a 60-residue proline-rich segment (Coutte,2003). *E. coli* YilG, a predicted IM-localized lipoprotein of unknown function also has a proline-rich tether that may be as long as 51 residues in length. Other proteins have much shorter predicted tethers. The Pal lipoprotein from *E. coli*, for instance, may have a tether of only two or three residues (PDB: 1EQ7, (Shu,2000)). These residues are thought to form a cap-like structure that stabilizes an Lpp trimer (Shu,2000). Together with the lipidated cysteine, this shortened tether may allow for Lpp to retain close proximity to the outer membrane while enhancing the stability of the membrane-peptidoglycan interaction that leads to the structural rigidity of the Gram-negative cell envelope. Longer tethers may impart upon a lipoprotein a larger radius of gyration such that they can retain membrane localization without compromising their ability to bind to and interact with a partner. CspA/BbCRASP-1, a host complement factor H (FH)-binding lipoprotein of B. burgdorferi, may require its long amino-terminal extension of 46 residues in order to extend fully beyond the dense layer of highly-expressed OspA/OspC lipoproteins coating the borrelial cell surface (Cordes,2005;Bunikis,2001). Truncation mutants of BbCRASP-1 indicate that the FH binding site is located at the extreme C-terminus, suggesting that this region of the protein could best be served projecting outward as far as practicable from the cell surface for optimal function (Kraiczy,2004;Cordes,2005).

Another mechanistic possibility for tether function is as a means for protein turnover upon changing environmental conditions, akin to PEST sequences in eukaryotic proteins (Rogers,1986;Wright,1999;Fink,2005). The unstructured nature of the tether may prove to be the ideal substrate for proteolytic cleavage of a given lipoprotein. *Trypanosoma brucei*, the African trypanosome, must rapidly exchange its GPI-anchored Variant Surface Glycoproteins (VSGs) with unrelated surface proteins as its life cycle transitions between its mammalian host and tsetse fly vector. These VSGs are proteolytically cleaved and released from the surface through the actions of a zinc metalloprotease and a phospholipase (Butikofer,2001;Gruszynski,2003;LaCount,2003;Grandgenett,2007). It would be easy to see how *B. burgdorferi* might likewise benefit from rapidly removing and exchanging immunodominant lipoproteins such as OspA and OspC from its surface during its tick-to-mammal transition.

The long unstructured tether domain may also be the location of a chaperone- or other partner-binding site. We consider such a possibility in Fig 15. Binding of another protein to the tether may induce the formation of a secondary structural element that permits recognition by an OM translocation system. This would be similar to the secretion mechanism of the *Yersinia pseudotuberculosis* effector YopE, where a disorder-to-order transition induced in its N-terminus by the Type III chaperone SycE permits its transport through the Type III needle apparatus (Rodgers,2008). It seems unlikely that the VSSL sequence represents a specific sequence-dependent target for binding by a chaperone as it was not found in the N-

terminus of any other *B. burgdorferi* lipoprotein. Unless OspA has a dedicated binding partner, the interaction of a chaperone with the tether may be governed by a sequence-independent interaction.

This study suggests that particular residues within the tether have important roles in surface localization, but the mechanism by which they promote OM translocation remains unclear. One possibility might involve their contribution to the overall flexibility of the N-terminus. Replacement of OspA residues 21-24 with alanines has no effect on OspA surface exposure, whereas substitution with glycines is detrimental. As glycine residues are less likely to allow formation of secondary structural elements (Chakrabartty,1991), OM translocation may in part be dependent on the ability of the tether to undergo some degree of conformational organization resulting in the adoption of a translocation-competent state. Transport of OMPs across the cytoplasmic membrane of bacteria has long been known to be dependent on the formation of secondary structure within the hydrophobic domain of the signal sequence (Emr,1983). The inability of the tether to be transported across the OM may therefore be due to an analogous problem arising from the presence of an overly flexible polyglycine tract.

Aside from structural functions, the tether of lipoproteins in Gram-negative bacteria contains information regarding membrane sorting. For *B. burgdorferi*, an organism containing a diverse array of lipoproteins exposed on its cell surface, there is very little in the way of a defined 'sorting signal' that is based on peptide sequence and easily identifiable upon comparison of the N-termini of the known surface

lipoproteins. It is for this reason that we previously hypothesized the existence of a default sorting pathway of lipoproteins in this bacterium (Schulze,2006). In the absence of a straightforward sorting signal, the directed targeting of proteins within bacteria necessitates some pliability on the part of the targeting/secretion machinery. Often, the signals for peptide localization are degenerate in nature with numerous exceptions to an accepted 'rule.' The Lol system for lipoprotein localization represents a pertinent example of a 'rule' with many exceptions. When it was first described two decades ago, an aspartic acid residue at position +2 of the mature lipoprotein was thought to be sufficient for retention of a lipoprotein in the inner membrane of *Escherichia coli* (Yamaguchi,1988). Subsequent studies revealed that an aspartic acid at this position is indeed a very potent inhibitor of the interaction between a mature lipoprotein and the LolCDE complex resident in the inner membrane and that this inhibition may be dependent on the atomic distance between its Cα carbon and the negative charge of its side chain (Hara,2003). Additionally, the presence of phosphatidylethanolamine in the *E. coli* membrane appears to contribute to the effectiveness of an Asp+2 avoidance signal. It is now known, however, that Asp at this position can be replaced with Asn, provided that an Asp residue appears at the +3 position. The *E. coli* lipoprotein AcrE (EnvC) contains such a signal and is thought to be localized to the cytoplasmic membrane (Seiffer,1993). Though not found in the *E. coli* genome, lipoproteins containing Phe, Pro, Trp, and Tyr at the +2 position and Asn at +3 will also be retained in the inner membrane (Seydel,1999;Terada,2001). Glycine at +2 was shown to partially retain a lipidated

form of MalE in the *E. coli* inner membrane but results in the transport of RlpE, which also has Gly at +2, to the inner leaflet of the outer membrane (Seydel,1999;Wu, 2006). As suggested by Seydel *et al*., the *E. coli* Lol system may accommodate the existence of ambiguous sorting signals to permit localization of a lipoprotein in both membranes.

More recent studies show that unlike the Enterobacteriaceae*, Pseudomonas aeruginosa* lipoproteins may depend on a '+3/+4-rule' with Lys-Ser as the primary inner membrane retention signal (Narita,2007). Our previous work excluded the possibility of either +2 or +3/+4 sorting rules for lipoproteins in *Borrelia* (Schulze,2006). Further investigation of lipoprotein sorting signals from more diverse organisms may reveal that each species either has different mechanisms to cope with the transport of these unique proteins or that sorting uses sequence-independent factors such as conformation to control localization, as has been suggested previously (Robichon,2003;Cullen,2004). Because we observed the same phenotype for the single residue deletions of both wild-type OspA and the mRFPΔ4 fusions, we can conclude that the information for membrane sorting is exclusive to the tether and not influenced in any way by the structural portion of the protein nor by the signal sequence, which remains unchanged for all constructs.

Though we show that tether length by itself is not the exclusive determinant of lipoprotein translocation across the outer membrane, it may play a role in combination with other signals. We show in this paper that removal of Leu24 from

OspA results in an OM translocation defect but that the leucine at this position is not absolutely required for proper surface localization. The placement of an alanine elsewhere within the tether can overcome the transport defect. Perhaps the critical VSSL region of OspA requires flanking regions of flexibility to function appropriately as a binding site. The removal of Leu24 has less of a defect than removal of Ser22/23 or Val21 and its role in translocation may therefore not be as critical as the other three residues. Addition of the alanine at position +10 may be sufficient to compensate for the function of the leucine at position +8.

Addition of C-terminal His-tags to both surface and subsurface lipoproteins suggests that some lipoproteins may traverse the *Borrelia* OM via a C-terminal first mechanism (Fig. 3D). Treatment of cells expressing these constructs with proteinase K revealed that only the His-tag (and six-residue linker) but not the mature portion of OspA was protease-accessible (i.e. surface exposed). It is unknown whether the translocation-impaired constructs are locked in a *bona fide* intermediate state of OM transport or whether addition of the His-tag results in the non-specific sampling of C-termini through OM channels. If the lipoproteins are indeed locked in an intermediate state of translocation, it may be possible to identify novel components of the OM transport machinery via crosslinking and coaffinity purification experiments.

The experiments performed in this paper represent further steps toward the goal of identifying regions and signals within lipoproteins that contribute to their ultimate placement within the bacterial cell envelope. We have identified one such region within the tether of a major virulence determinant of *Borrelia burgdorferi* that is

critical for transport of the lipoprotein to the cell surface. Though different mechanisms and signals may be used for lipoprotein transport by different species of bacteria, the common feature of a lengthy disordered N-terminus may reveal insights into a shared property for these specialized proteins.

Experimental procedures

Bacterial strains and growth conditions. OspA- strain *B. burgdorferi* B313 was used for expression of all constructs(Sadziene,1993). B313 is a clone of type strain B31 (ATCC 35210) and contains circular plasmids cp26, cp32-1, cp32-2/7, cp32-3 and the single linear plasmid lp17 (Zuckert,1999; Zuckert,2004). *B. burgdorferi* were cultured in liquid or solid BSK-II medium at 34°C under 5% CO2 (Barbour,1984). *E. coli* strains TOP10 (Invitrogen, Carlsbad, CA) and XL10-Gold (Stratagene) were used for recombinant plasmid construction and propagation and grown in Luria Bertani (LB) broth or on LB agar (Difco).

Lipoprotein Fusion and Point Mutants. All mutations made in this study were constructed in pBSV2(Stewart,2001), an *E. coli-B. burgdorferi* shuttle vector conferring kanamycin resistance. Expression of all lipoprotein constructs is under the control of the constitutive *B. burgdorferi* flagellin *flaB* promoter (P*flaB*). Point mutations were generated by using the QuikChange-II XL site-directed mutagenesis kit (Stratagene). pCSY6a-linkerHis is a pBSV2-derived plasmid containing an epitope-tagged copy of wild-type OspA under the control of the *flaB* promoter. A C-

Table 7. Bacterial Strains and Plasmids Used in this Study

Strain/Plasmid		Description	Source/Reference
Strains			
	Borrelia burgdorferi		
	B313	Clone of B31 ATCC 35210 (cp26, cp32-1, cp32-2/7, cp32-3 and lp17). OspA$^-$.	(Sadziene,1995)
	Escherichia coli		
	Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
	XL-10 Gold	Tet^r D*(mcrA)183* D*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* The [F' *proAB lacI^q*ZD*M15 Tn10* (Tet^r) Amy Cam^r	Stratagene
Plasmids			
	pRJS0998	pBSV2:PflaBospA	(Schulze,2006)
	pRJS1029	pBSV2:PflaBospAΔL24	(Schulze,2006)
	pRJS1030	pBSV2:PflaBospA28ΔL24-mRFP1	(Schulze,2006)
	pRJS1036	pBSV2:PflaBospA22(ED)	This study
	pRJS1040	pBSV2:PflaBospAΔL24ΩA27	This study
	pRJS1041	pBSV2:PflaBospA28ΔL24-mRFPΔ4	This study
	pRJS1077	pBSV2:PflaBospA28-mRFPΔ4	This study
	pRJS1078	pBSV2:PflaBospAΔN20	This study
	pRJS1130	pBSV2:PflaBospA(Ala)4	This study
	pRJS1131	pBSV2:PflaBospA(Gly)4	This study
	pRJS1140	pBSV2:PflaBospAΔS22/23-His	This study
	pRJS1141	pBSV2:PflaBospAΔL24-His	This study
	pRJS1200	pBSV2:PflaBospAΔK18	This study
	pRJS1201	pBSV2:PflaBospAΔQ19	This study
	pRJS1202	pBSV2:PflaBospAΔV21	This study
	pRJS1203	pBSV2:PflaBospAΔS22/23	This study
	pRJS1204	pBSV2:PflaBospAΔD25	This study
	pRJS1205	pBSV2:PflaBospAΔE26	This study
	pRJS1206	pBSV2:PflaBospAΔK27	This study
	pRJS1207	pBSV2:PflaBospAΔN28	This study
	pCSY6a-linkerHis	pBSV2:PflaBospA-His	This study

Table 8. Oligonucleotides used in this study.

Name	Sequence	Description
1029-fwd	5’ TGTAAGCAAAATGTTAGCAGCGACGAGAAAAAC 3’	OspAΔL24 forward mutagenic primer
1029-rev	5’ GTTTTTCTCGTCGCTGCTAACATTTTGCTTAC 3’	OspAΔL24 reverse mutagenic primer
1040-fwd	5’ GCAGCGACGAGGCTAAAAACAGCGTTTCAG 3’	OspAΔL24ΩA27 forward mutagenic primer
1040-rev	5’ GAAACGCTGTTTTTAGCCTCGTCGCTGCTAACATTTTG 3’	OspAΔL24ΩA27 reverse mutagenic primer
1041-fwd	5’ TTAGCAGCGACGAGAAAAACGACGTCATCAAGG 3’	OspA28ΔL24:mRFPΔ4 forward mutagenic primer
1041-rev	5’ CTTGATGACGTCGTTTTTCTCGTCGCTGCTAACATTTTG 3’	OspA28ΔL24:mRFPΔ4 reverse mutagenic primer
1077-fwd	5’ AAATGTTAGCAGCCTTGACGAGAAAAACGACGTCATC 3’	OspA28:mRFPΔ4 forward mutagenic primer
1077-rev	5’ TCGTTTTTCTCGTCAAGGCTGCTAACATTTTGCTTAC 3’	OspA28:mRFPΔ4 reverse mutagenic primer
1078-fwd	5’ GCCTTAATAGCATGTAAGCAAGTTAGCAGCCTTGAC 3’	OspAΔN20 and OspA28ΔN20:mRFPΔ4 forward mutagenic primer
1078-rev	5’ GTCAAGGCTGCTAACTTGCTTACATGCTATTAAGG 3’	OspAΔN20 and OspA28ΔN20:mRFPΔ4 reverse mutagenic primer
1120-fwd	5’ AGCATGTAAGCAAAATGCAGCTGCAGCTGACGAGAAAAAC 3’	OspA(Ala)4 forward mutagenic primer
1120-rev	5’ TTTTTCTCGTCAGCTGCAGCTGCATTTTGCTTACATGC 3’	OspA(Ala)4 reverse mutagenic primer
1121-fwd	5’ ATGTAAGCAAAATGGAGGCGGAGGCGACGAGAAAAAC 3’	OspA(Gly)4 forward mutagenic primer
1121-rev	5’ GTTTTTCTCGTCGCCTCCGCCTCCATTTTGCTTACATG 3’	OspA(Gly)4 reverse mutagenic primer
1200-fwd	5’ TTAGCCTTAATAGCATGTCAAAATGTTAGC 3’	OspAΔK18 forward mutagenic primer
1200-rev	5’ CGTCAAGGCTGCTAACATTTTGACATGCTATTAAG 3’	OspAΔK18 reverse mutagenic primer
1201-fwd	5’ CCTTAATAGCATGTAAGAATGTTAGCAGCCTTGAC 3’	OspAΔQ19 forward mutagenic primer
1201-rev	5’ GTCAAGGCTGCTAACATTCTTACATGCTATTAAGG 3’	OspAΔQ19 reverse mutagenic primer
1202-fwd	5’ CCTTAATAGCATGTAAGCAAAATAGCAGCCTTGAC 3’	OspAΔV21 forward mutagenic primer
1202-rev	5’ GTCAAGGCTGCTATTTTGCTTACATGCTATTAAGG 3’	OspAΔV21 reverse mutagenic primer
1203-fwd	5’ GCAAAATGTTAGCCTTGACGAGAAAAACAGCGTTTCAGTAG 3’	OspAΔS22 forward mutagenic primer
1203-rev	5’ AAACGCTGTTTTTCTCGTCAAGGCTAACATTTTGC 3’	OspAΔS22 reverse mutagenic primer
1204-fwd	5’ TGTTAGCAGCCTTGAGAAAAACAGCGTTTCAGTAG 3’	OspAΔD25 forward mutagenic primer
1204-rev	5’ AAACGCTGTTTTTCTCAAGGCTGCTAACATTTTGC 3’	OspAΔD25 reverse mutagenic primer
1205-fwd	5’ TGTTAGCAGCCTTGACAAAAACAGCGTTTCAGTAG 3’	OspAΔE26 forward mutagenic primer
1205-rev	5’ AAACGCTGTTTTTGTCAAGGCTGCTAACATTTTGC 3’	OspAΔE26 reverse mutagenic primer
1206-fwd	5’ TGTTAGCAGCCTTGACGAGAACAGCGTTTCAGTAG 3’	OspAΔK27 forward mutagenic primer
1206-rev	5’ GGCAAATCTACTGAAACGCTGTTCTCGTCAAGG 3’	OspAΔK27 reverse mutagenic primer
1207-fwd	5’ GCAGCCTTGACGAGAAAAGCGTTTCAGTAGATTTG 3’	OspAΔN28 forward mutagenic primer
1207-rev	5’ ATCTACTGAAACGCTTTTCTCGTCAAGGCTGCTAAC 3’	OspAΔN28 reverse mutagenic primer

terminal six residue linker (Pro-Gly-Gly-Ser-Gly-Ala) precedes a hexhistadine tag. The $OspA_{\Delta S22}$ and $OspA_{\Delta L24}$ mutations were introduced into this plasmid by site-directed mutagenesis. All mutations and constructs were verified by sequencing (Center for Genetic Medicine, Genomics Core Facility, Northwestern University Medical Center, Chicago, IL). *B. burgdorferi* cells were transformed by electroporation using 1-5 μg of plasmid DNA using established protocols (Stewart,2001). Transformants were selected in solid BSK-II containing 200 μg/ml kanamycin, with three independent clones expanded in selective liquid BSK-II. Plasmid profiles were determined by PCR using plasmid-specific oligonucleotide primers (Purser and Norris, 2000, Proc Natl Acad Sci U S A, 97, 13865-70; Labandeira-Rey,2001). The resulting recombinant strains are listed in Table 7.

Protein Gel Electrophoresis and Immunoblot analysis. Proteins were separated by sodium dodecyl sulfate-12% polyacrylamide electrophoresis (SDS-PAGE) and visualized by Coomassie blue staining. For immunoblots, proteins were electrophoretically transferred to nitrocellulose membranes (Immobilon-NC, Millipore) using a Transblot-SD Semi-Dry Transfer Cell (Bio-Rad) as described (Schulze, 2006). Membranes were blocked and incubated with antibodies in 5% dry milk, 20 mM Tris-500 mM NaCl, 0.05% Tween 20. Antibodies used were anti-mRFP1 rabbit polyclonal antiserum (1:1,000 dilution), anti-OppAIV rabbit polyclonal antiserum (1:100 dilution) (Bono et al., 1998, Microbiology, 144 , 1033-44), or mouse monoclonal antibodies against *B. burgdorferi* OspA (1:25 dilution,

H5332) (Barbour,1983) and FlaB (1:25 dilution, H9724) (Barbour et al., 1986, Infect Immun, 52, 549-54). Secondary antibodies were alkaline phosphatase-conjugated goat-anti-rabbit IgG (H+L) or goat-anti-mouse IgG (H+L) (Bio-Rad). Alkaline phosphatase substrates were 1-Step NBT/NCIB (Pierce) for colorimetric and CDP-Star (Amersham Biosciences) for chemiluminescent detection.

Protease and Antibody Accessibility Assays. Spirochetes were harvested, washed and resuspended in phosphate-buffered saline containing 5mM MgCl2 (PBS+Mg) as described (Barbour,1984). To assess protein surface exposure by protease accessibility, intact *B. burgdorferi* cells were treated *in situ* with 200 µg/ml proteinase K (Invitrogen) as described (Bunikis and Barbour, 1999, Infect Immun, 67, 2874-83). Cells were analyzed by epifluorescence microscopy using a Nikon Eclipse E600 microscope fitted with a Texas Red HYQ filter blocks and a QImaging Micropublisher Digital CCD color camera. Digital images were processed using Adobe Photoshop CS and ImageJ version 1.33u (NIH) for Macintosh on an Apple Macbook.

Membrane and Protein Fractionations. Membrane fractionation of *Borrelia* was performed as described earlier(Skare et al., 1995, J Clin Invest, 96, 2380-92;Schulze, 2006).

Lipoprotein Sequence Analysis and Protein Disorder Prediction. The 90 experimentally-verified *E. coli* K12 lipoproteins from (Tokuda,2007) and the 127 *B. burgdorferi* B31 lipoproteins identified as being part of the 'liposet' identified in (Setubal,2006) were used for sequence comparisons. LogoBar (Perez-Bercoff,2006) was used for creation of Figure 4. Predictions for protein disorder were performed using the VSL2B algorithm on the web-based DisProt site (http://www.ist.temple.edu/disprot/predictorVSL2.php). Control cytoplasmic and periplasmic proteins from *E. coli* were selected from a pool identified as such by both Swiss-Prot and from proteomic analysis performed in (Lopez-Campistrous,2005).

Chapter IV. Localization of Lipoproteins lp6.6 and BB0323 to Subsurface Membrane Compartments

Abstract

Lipoproteins are known to play numerous important roles in the physiology and virulence of bacteria. Most diderm organisms (those with two membranes) sort lipoproteins within the boundaries of the periplasmic space. *Borrelia burgdorferi*, the etiologic agent of Lyme disease, is unique among diderms because it has a large number of lipoproteins that are additionally localized to its cell surface. The process of differential sorting of bacterial lipoproteins has been studied for thirty years in *Escherichia coli*, yet only recently have attempts been made at understanding the lipoprotein sorting process in *Borrelia*. We have previously characterized the determinants of the *B. burgdorferi* surface lipoprotein OspA. In an effort to better understand the characteristics of native lipoproteins that reside in all three membrane compartments, we chose to analyze two additional lipoproteins with putative periplasmic localization. Here, we characterize lp6.6 (BBA62) and BB0323 and show that fusions of each to a red fluorescent protein (mRFPΔ4) localize predominantly to the periplasmic faces of the outer and inner membranes, respectively. We introduce the technique of plasmolysis to *Borrelia* to show that it may be useful in determining membrane localization of fluorescent lipoproteins. These fluorescent fusions can serve as the basis for further studies for elucidating the mechanisms whereby *B. burgdorferi* selectively localizes lipoproteins within its cell envelope.

Introduction

The sorting of lipoproteins in *Escherichia coli* has been thoroughly dissected using numerous representative lipoproteins to illustrate the importance of the residue at the +2 position (and to a lesser extent the +3 position) for inner membrane (IM) retention (Narita,2004;Tokuda,2007). Many of the original experiments used fusions of the β-galactosidase reporter (β-gal) to the peptidoglycan-binding lipoprotein Lpp to determine the effect of N-terminal mutagenesis on lipoprotein sorting (Yamaguchi,1988). Additional experiments have used alternative reporters such as the maltose-binding protein (MalE), bacteriophage T5-encoded lipoprotein (Llp), and monomeric red fluorescent protein (mRFP1) (Seydel,1999;Robichon,2003;Lewenza,2006). Through detailed comparison of sequences and analysis of mutant phenotypes, it was determined that an aspartic acid at the +2 position behaves as a strong Lol-avoidance signal, possibly because of interactions between its negatively-charged side chain and phosphatidylethanolamine phospholipids in the inner membrane (IM) (Hara,2003). The strength of IM retention can be modified by the identity of the amino acid at the +3 position (Seydel,1999)

Characterization of lipoprotein sorting rules in other bacteria has lagged far behind the studies done in *E. coli* and other members of the family Enterobacteriaceae. It is now becoming increasingly apparent that different organisms may use very different signals to govern localization of their constituent lipoproteins. *Pseudomonas aeruginosa*, for example may rely on the identity of residues at the +3/+4 positions

(Narita,2007;Tanaka,2007). The *Yersinia pestis* lipoprotein YscJ is anchored into the IM regardless of the identity of the residues in the +2/+3 positions, suggesting an alternative signal for lipoprotein retention in that organism (Silva-Herzog,2008).

We have already performed preliminary analyses on the sorting of the major surface lipoprotein of *Borrelia burgdorferi*, OspA (Schulze,2006;Schulze,2009)). The use of fluorescent fusions to the OspA tether result in the trafficking of the reporter protein to the cell surface. We have demonstrated the ability to restrict localization of the reporter to all three compartments using site-directed mutagenesis. As OspA is only native to the cell surface, however, we wished to make similar reporter fusions to proteins known to be native to the other two membrane compartments (the periplasmic faces of the inner and outer membranes). Reports of proteins localizing to these compartments in *B. burgdorferi* are sparse and based only on predictions of protein function.

OppAIV is one of five homologues of a periplasmic binding protein for an oligopeptide permease (Bono,1998). We conclusively showed that it localizes strictly to the *Borrelia* inner membrane. However, a fusion of the N-terminal 31 amino acids (the signal II peptide and predicted tether) to mRFP1 was sorted to the cell surface (Schulze,2006). To better understand the sorting rules of *Borrelia burgdorferi*, we sought to identify additional candidate IM and OMIL (**o**uter **m**embrane, **i**nner-**l**eaflet)-resident lipoproteins with which we could generate reporter fusions.

Aside from OppAIV, only IpLA7 has been shown by our laboratory to be an IM-specific lipoprotein (vonLackum,2007). Our attempts at creating an IpLA7-mRFP fusion have been hampered by cloning difficulties. A recent transposon mutagenesis screen of the *B. burgdorferi* B31 genome identified a gene, *BB0323*, that when disrupted by the transposon, resulted in severe membrane blebbing. A LysM domain was predicted to be located near the C-terminus of the protein encoded by *BB0323*. LysM domains are frequently associated with binding to peptidoglycan cell wall structures (Bateman,2000;Buist,2008). Because *B. burgdorferi* is thought to have a cell wall closely associated with the IM (rather than the OM, as is common for most Gram-negative organisms), we hypothesized that BB0323 may be an IM-localized lipoprotein with a function analogous to Lpp or Pal of *E. coli*.

No lipoproteins have been definitively characterized as OMIL lipoproteins in *Borrelia*. A previous analysis and characterization of the *BBA62* gene product provided conflicting localization data, with membrane fractionation showing that lp6.6 was predominantly localized to the protoplasmic cylinder but Triton X-114 phase separation showing localization to the outer membrane (Lahdenne,1997). Protease accessibility suggested that the protein was subsurface; however, these studies were performed in the pathogenic *B. burgdorferi* strain B31, known to express numerous surface-localized Osp proteins. Previous experiments have shown that these Osps may hinder protease and antibody accessibility to underlying smaller,

surface-exposed proteins or protein loops such as those found on the OM porin p66 (Bunikis,1999).

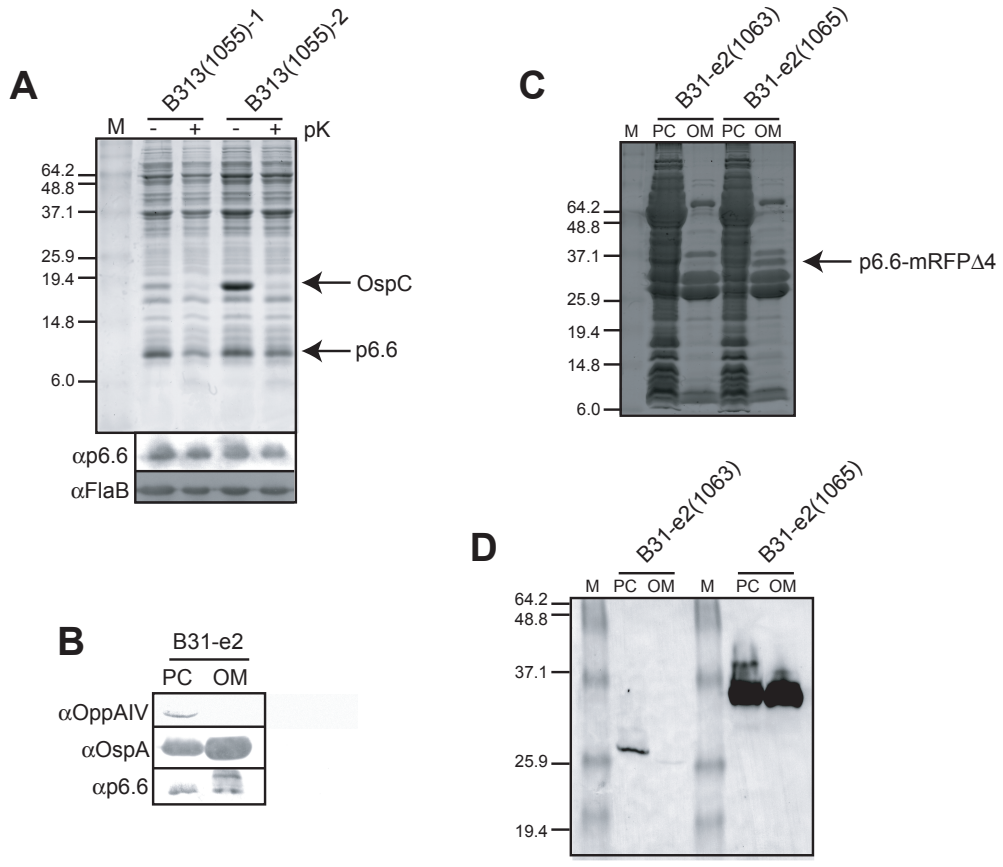
In this study, we used an OspAB-deficient *B. burgdorferi* strain (B313) to verify protease inaccessibility of lp6.6. We show that lp6.6 is indeed a subsurface lipoprotein. Fusions of a red fluorescent reporter to both lp6.6 and BB0323 indicate a periplasmic location of each, with the former localizing to the OM and the latter to the IM. We also show a potential role for plasmolysis in the determination of subsurface membrane localization of fluorescently-labeled lipoproteins.

Results

lp6.6 (BBA62) localizes to the inner leaflet of the outer membrane

To definitively determine the localization of lp6.6, we used a combination of proteinase K accessibility and isopycnic sucrose density gradient centrifugation to reveal the true membrane topology of this lipoprotein. We cloned the *bba62* locus (including its native promoter) into pBSV2, an *E. coli – B. burgdorferi* shuttle vector (Stewart,2001) and expressed the protein in *B. burgdorferi* strain B313, which is missing the linear plasmid containing the *bba62* gene (lp54) (Sadziene,1995). Treatment of cells expressing lp6.6 with 200μg/ml proteinase K revealed that p6.6, like the control periplasmic flagellar protein FlaB, is not sensitive to the protease (Fig. 17A). Because strain B313 is missing lp54, it is also missing the gene encoding OspA that is normally used as a surface-localized control protein. We therefore used

Figure 17. Localization of lp6.6 and BB0323P25:mRFPΔ4. (A) Coomassie-stained gel shows whole cell lysates from two separate clones of pK-treated and – untreated *B. burgdorferi* strain B313 expressing lp6.6 from its native locus on a shuttle plasmid (pRJS1055). Note the variable expression of OspC between the two clones but consistent profiles of other proteins. OspC is degraded by proteinase K treatment but lp6.6 and FlaB are not. (B) Membrane fractionations from wild-type B31-e2 show that lp6.6 has a clear association with the OM, similar to OspA. OppAIV is included as a strict IM-control. (C) Coomassie-stained gel of membrane fractions from B31-e2 expression either BB0323P25:mRFPΔ4 (off of pRJS1063) or lp6.6:mRFPΔ4 (off of pRJS1065). The lp6.6 fusion is clearly visible in the OMV fraction. (D) Western blot analysis of above fractions show clear confirmation that the lp6.6 fusion fractionates with the OM and BB0323P25:mRFPΔ4 is found only in the IM (protoplasmic cylinder) fraction.


A
B313(1055)-1
B313(1055)-2
M
-
+
-
+
pK
64.2
48.8
37.1
25.9
19.4
14.8
6.0
OspC
p6.6
αp6.6
αFlaB
B
B31-e2
PC
OM
αOppAIV
αOspA
αp6.6
C
B31-e2(1063)
B31-e2(1065)
M
PC
OM
PC
OM
64.2
48.8
37.1
25.9
19.4
14.8
6.0
p6.6-mRFPΔ4
D
B31-e2(1063)
B31-e2(1065)
M
PC
OM
M
PC
OM
64.2
48.8
37.1
25.9
19.4

OspC instead. As seen on the Coomassie-stained gel, OspC is almost entirely digested whereas p6.6 is not. Note that lp6.6 migrates with an apparent mass of 10kD on SDS-PAGE gels (Lahdenne,1997;Katona,1992). This finding verifies previous reports that lp6.6 has a subsurface localization.

To determine which membrane lp6.6 is anchored into, we treated B31-e2 cells in a hypotonic citrate buffer and performed sucrose density gradient ultracentrifugation to separate outer membrane vesicles from the protoplasmic cylinder. Fig. 17B shows that unlike OppAIV, lp6.6 maintains a significant association with the OMV fraction, similar to that of OspA, a known surface lipoprotein. Together with its resistance to proteinase K digestion, we conclude that lp6.6 is a *bona fide* OMIL lipoprotein.

An in-frame fusion of mRFPΔ4 (Schulze,2009) to the C-terminus of lp6.6 was cloned into pBSV2, producing pRJS1065. This plasmid was used to transform *B. burgdorferi* strain B31-e2 and resulted in the production of a protein of approximately 34kDa in size (Fig. 17C). Membrane fractionation clearly shows an association of this fusion protein with the OMV fraction in both a Coomassie-stained gel of the fractions (Fig. 17C) and in a Western blot using antibodies against mRFP1 (Fig. 17D, right). Additionally, the OMV fraction itself was noticeably pink in color (data not shown). Together, these results demonstrate an OMIL localization of lp6.6.

A BB0323-mRFPΔ4 fusion localizes to the inner membrane of B. burgdorferi

As the predicted *BB0323* gene product contains a C-terminal LysM domain hypothesized to play a critical role in peptidoglycan binding, we opted to not fuse mRFPΔ4 to the carboxy-terminus of the full-length protein. We instead fused the N-terminal 25 residues (up to Pro25) to mRFPΔ4 to determine whether BB0323 would be likely retained in the IM or sorted to the OMIL. This fusion was cloned into pBSV2, producing pRJS1063. Strains of B31-e2 that had been electrotransformed with this plasmid did not produce this protein in large amounts as seen on a Coomassie-stained gel of membrane fractions (Fig. 17C). However, unlike the fractions of the lp6.6-mRFPΔ4 fusion, the protoplasmic cylinder fraction was bright pink in color whereas the OMV fraction was white (data not shown). A confirmatory Western blot (Fig. 17D, left) shows that the BB0323 fusion fractionates exclusively with the PC fraction. This is the first indication that BB0323 may, like OppAIV and IpLA7, be an IM-localized lipoprotein.

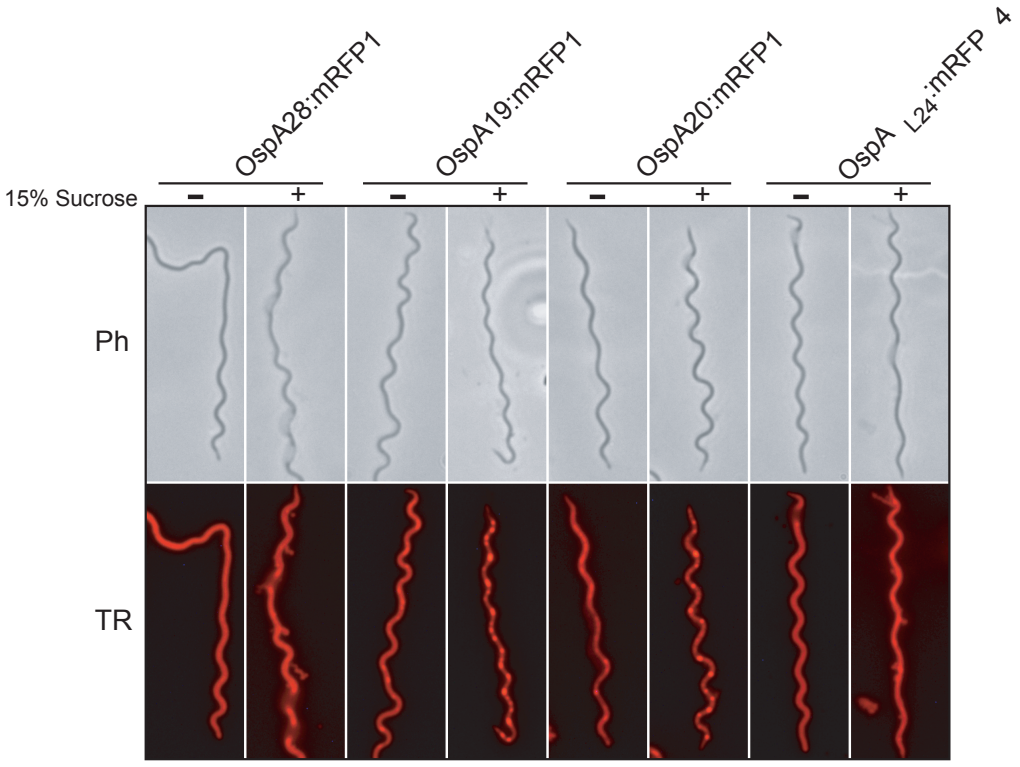
Plasmolysis of B. burgdorferi

Though reliable and consistent, the fractionation of *B. burgdorferi* membranes is a laborious and time-consuming process. The process is no easier in other diderm bacteria. A technique involving plasmolysis of the inner and outer membranes has recently been applied for the study of lipoprotein localization and used with success in various members of the Enterobacteriaceae as well as in *Pseudomonas aeruginosa*.

Treatment of *E. coli* with 15% sucrose causes a localized retraction of the inner membrane from the OM-associated peptidoglycan cell wall to form so-called "plasmolysis bays" (Cook,1987;Mulder,1993;Lewenza,2006). Red fluorescent proteins that have been retained in the inner membrane are therefore easily seen in such cells as they follow the contour of the retracted membrane when visualized under fluorescence microscopy. Recent work has shown that treatment of *Pseudomonas aeruginosa* with 0.5M NaCL results in the same phenomenon (Lewenza,2008). We sought to determine whether plasmolysis might be a useful tool in directly visualizing membrane localization of lipoproteins in *Borrelia.*

We chose four previously localized lipoprotein-reporter fusions from a previous study (OspA28:mRFP1 [OM], OspA19:mRFP1 [IM], OspA20:mRFP1 [IM], and $OspA_{\Delta L24}$:mRFPΔ4 [OM], (Schulze,2009)) and expressed these constructs in *B. burgdorferi* strain B31-e2. Treatment of cells with 0.5M NaCl showed little difference between the cells when viewed under fluorescence microscopy (data not shown). However, treatment with 15% sucrose showed a faint but reproducible punctate pattern along the length of the spirochete for those constructs known to be localized to the inner membrane (Fig. 18). No pattern was seen for cells treated with PBS-Mg. We conclude that plasmolysis of *B. burgdorferi* may be a suitable supplementary test for confirmation of lipoprotein membrane localization.

Figure 18. Plasmolysis assay. B31-e2 cells expressing one of four constructs with known membrane localization were treated with either PBS or 15% sucrose. As can be seen in lanes 4 and 6, punctate staining of mRFP corresponds to the formation of plasmolytic bays for the two IM-localized reporter proteins. No such staining is seen for either OspA28:mRFP1 nor $OspA_{\Delta L24}$:mRFPΔ4. Abbreviations: Ph (Phase contrast), TR (Texas Red epifluorescence filter).


OspA28:mRFP1
OspA19:mRFP1
OspA20:mRFP1
OspA L24:mRFP 4
15% Sucrose
-
+
-
+
-
+
-
+
Ph
TR

Discussion

We show here evidence that lp6.6 and BB0323, two *B. burgdorferi* lipoproteins with previously unknown or unconfirmed localization, are sorted to the periplasmic faces of the outer and inner membranes, respectively. Fusions of the full-length lp6.6 or the N-terminus of BB0323 to mRFPΔ4 localize within the periplasmic space and can now be further investigated for the presence of possible sequence-dependent and –independent sorting signals that *B. burgdorferi* uses as a basis for their localization. We also provide evidence that osmotic shock of *Borrelia* cells can induce the formation of plasmolytic bays that may aid in the expedient determination of membrane localization for fluorescently-tagged lipoproteins.

BBA62 (lp6.6), like OspA, is a lipoprotein that has been shown in numerous studies to be significantly downregulated during the growth of *B. burgdorferi* in the mammalian host (Lahdenne,1997;Yang,2000;Liang,2002;Brooks,2003). This downregulation appears to be RpoS-dependent in nature (Caimano,2005;Caimano,2007). A potential role for this protein has not been elucidated. However, the presence of *BBA62* on the same linear plasmid (lp54) as *ospA* and similar patterns of regulation for lp6.6 and OspA may be indicative of a potential role in the midgut colonization of the *B. burgdorferi* arthropod vector, *Ixodes scapularis*. We show that this protein localizes preferentially to the OMIL. It is tempting to speculate that lp6.6 might be somehow connected to OM translocation

of OspA, considering the genes encoding each are located on the same plasmid and are up- and downregulated by similar environmental cues. Additional studies are required to determine whether such an association of two proteins on opposite leaflets of the OM might exist.

Very little is known about the function of the BB0323 protein. Recent studies show that it is a highly immunogenic lipoprotein and that IgG antibodies to BB0323 are developed at both early and late stages of human infection with *B. burgdorferi* (Nowalk,2006; Tokarska-Rodak,2008). Insertion of the Himar1 *mariner* transposon into the *BB0323* open reading frame results in serious membrane stability problems for the cell (Stewart,2004). This would seem to indicate a role for BB0323 in the maintenance of membrane integrity. The discovery of a LysM domain near the C-terminus may provide a hint for this protein's function. If indeed BB0323 has a purely structural function similar to that of Braun's lipoprotein (Lpp), this may account for the finding that it is not reciprocally regulated like other immunogenic lipoproteins and explain why it is found at both early and late stages of *B. burgdorferi* infection in humans. To avoid disrupting a potential peptidoglycan-binding role for BB0323, we chose not to fuse mRFPΔ4 to the full-length protein. A further reason for avoiding a full-length fusion is that BB0323 has been suggested to be a substrate of a *B. burgdorferi* C-terminal protease, CtpA (Ostberg,2004). We therefore opted to make a short fusion of the signal peptide and portion of the predicted tether to mRFPΔ4 and found that this fusion ($BB0323_{P25}$:mRFPΔ4) is retained exclusively in

the borrelial IM. Longer fusions of the BB0323 N-terminus to mRFPΔ4 may be warranted to conclusively determine the localization of the full-length protein.

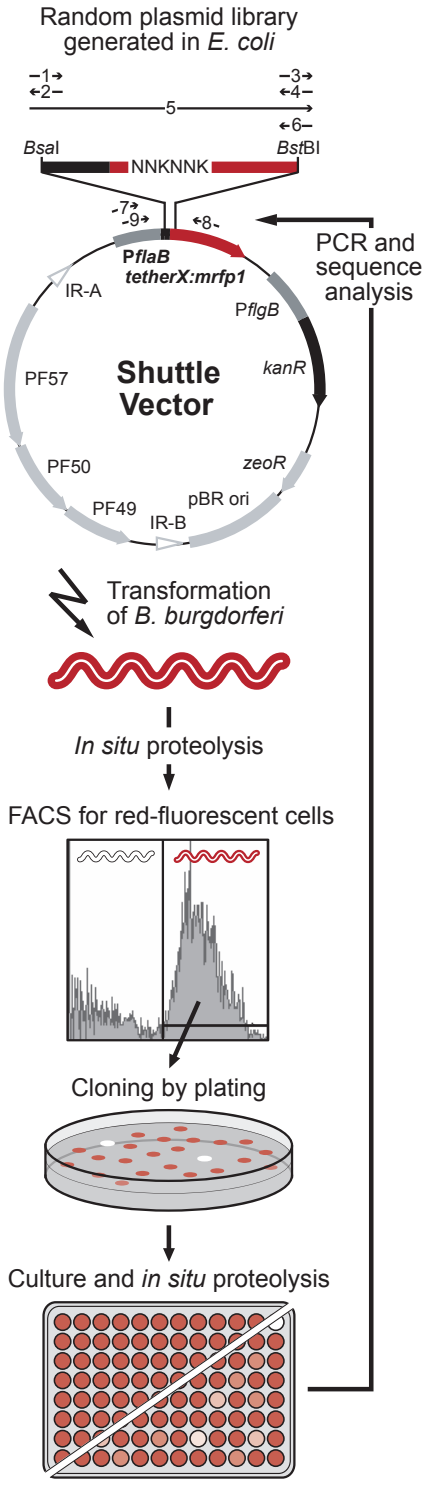
Regardless of the functions for lp6.6 or BB0323, the finding that these lipoproteins may preferentially be targeted to the IM or OMIL suggests that the transport of lipoproteins to the spirochetal cell surface may involve retention "checkpoints" in both membranes. After transport through the Sec apparatus, a strict decision appears to be made as to whether a lipoprotein is retained in the IM or shuttled across the periplasm to the OM. At the OMIL, a second recognition step occurs, determining whether the protein is further transported across the OM or is retained on the periplasmic face of the OM. The existence of such a system may, in part, explain our previous finding that single-residue deletions within the OspA tether are transported to the OM as efficiently as wild-type, but are unable to be secreted across the membrane (Schulze,2006;Schulze,2009). A failed recognition step at the OMIL may prevent transport from occurring and further investigation is needed such that a putative OM translocation complex might be identified.

Together with our previous localization of OspA28:mRFPΔ4 and fusions created in this study, we have now isolated fluorescent lipoprotein fusions to the three possible membrane compartments of *B. burgdorferi*: BB0323 to the IM, lp6.6 to the OMIL, and OspA to the surface. This is important, because knowledge of lipoproteins that are naturally found in each of these three locations will help identify other

lipoproteins that may be targeted to the same position within the cell. This information will aid in the understanding of *B. burgdorferi* membrane architecture and may provide hints regarding the pathogenicity of this bacterium.

The fluorescent reporters will enable us to further study the primary sequence requirements for lipoprotein localization in this organism. One approach might involve the use of a random mutagenesis / fluorescence-activated cell sorting (FACS) screen (as diagrammed in Fig 19). In such a screen, random mutagenesis would be performed on the N-terminal tether of a known surface-localized protein (i.e. Osp28:mRFP1). A library of mutants would be used to transform *B. burgdorferi*, and cells grown in batch would be subjected to multiple rounds of FACS. The initial rounds would remove those cells not expressing a red-fluorescent protein. Subsequent rounds would be preceded by *in situ* proteolysis with proteinase K, to cleave all surface proteins from the bacterium. Cells remaining red fluorescent would theoretically contain a mutation resulting in a subsurface localization of the reporter and could be collected by FACS. These cells could then be further examined by isolation of single colonies and examination of periplasmic localization via a combination of membrane fractionation and the plasmolysis assay described above. DNA sequence analysis would allow for the identification of the mutation. In theory, such a screen would allow for a rapid and exhaustive search of sequences which permit retention at the periplasmic faces of the inner and outer membranes. A similar screen that starts with IM and OMIL-retained lipoproteins (i.e. the BB0323 and p6.6

Figure 19. FACS screen for lipoprotein localization. This diagram describes the fluorescence-based cell sorting assay proposed in the text. The randomly-mutagenized plasmid library is generated in *E. coli* and used to transform *B. burgdorferi*. FACS discards cells not expressing red fluorescent protein. The cells are then treated with proteinase K to cleave known surface proteins (including surface-localized mRFP1). If the cells remain fluorescent, it is likely that the mutations introduced a subsurface retention signal and these cells can be further examined by traditional methods.


Random plasmid library
generated in E. coli
1
2
3
4
5
6
BsaI
BstBI
NNKNNK
7
9
8
PflaB
tetherX:mrfp1
IR-A
PflgB
PF57
Shuttle
Vector
kanR
PF50
zeoR
PF49
pBR ori
IR-B
PCR and
sequence
analysis
Transformation
of B. burgdorferi
In situ proteolysis
FACS for red-fluorescent cells
Cloning by plating
Culture and in situ proteolysis

fusions identified in this study) could assay for mutations permitting surface exposure of the reporter. Results from such experiments would provide valuable data on the sequence-dependent requirements of lipoprotein sorting for *B. burgdorferi*.

Materials and Methods

Plasmids and Strains

B. burgdorferi strains B31-e2 and B313 have been described previously (Schulze,2006). See Table 9 for a list of strains and plasmids used in this study. Expression of *BBA62* in B313 was achieved by amplifying the *BBA62* gene and 1kb of flanking sequence with primers 1055-fwd/rev and cloning into the *B. burgdorferi – E. coli* shuttle vector pBSV2 to create pRJS1055. The *BB0323* locus was amplified in the same fashion using primers 1057-fwd/rev to create pRJS1057. Fusion of full length-*lp6.6* or $BB0323_{P25}$ to *mRFPΔ4* was performed using sequence-overlap extension (SOE, (Horton,1990) and pRJS1041 as the starting plasmid template. pRJS1041 contains the gene encoding the OspA28:mRFPΔ4 fusion under the control of the *flaB* promoter. The OspA28 portion of the gene was replaced by SOE with either full-length *lp6.6* or $BB0323_{P25}$ using flanking primers BamPflaB-fwd (5') and 1055-rev (3') as above. Internal primer pairs used were 1063-fwd/rev for *BB0323* or 1065-fwd/rev for *lp6.6* to generate plasmids pRJS1063 or pRJS1065, respectively. Electrotransformation and growth of *B. burgdorferi* was done as previously described (Schulze,2006).

Table 9. Bacterial Strains and Plasmids Used in this Study

Strain/Plasmid		Description	Source/Reference
Strains			
	Borrelia burgdorferi		
	B31-e2	Clone of B31 ATCC 35210 (cp26, cp32-1, cp32-3, cp32-4, lp17, lp38, lp54)	(Babb,2004)
	B313	Clone of B31 ATCC 35210 (cp26, cp32-1, cp32-2/7, cp32-3 and lp17). OspA$^-$.	(Sadziene,1995)
	Escherichia coli		
	Top10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G	Invitrogen
Plasmids			
	pRJS1009	pBSV2:PflaBospA28:mRFP1	(Schulze,2006)
	pRJS1014	pBSV2:PflaBospA19:mRFP1	(Schulze,2006)
	pRJS1016	pBSV2:PflaBospA20:mRFP1	(Schulze,2006)
	pRJS1041	pBSV2:PflaBospA28ΔL24-mRFPΔ4	(Schulze,2009)
	pRJS1055	pBSV2: lp6.6locus	This study
	pRJS1057	pBSV2:BB0323locus	This study
	pRJS1063	pBSV2:PflaBBB0323P25:mRFPΔ4	This study
	pRJS1065	pBSV2:PflaBlp6.6: mRFPΔ4	This study

Table 10. Oligonucleotides used in this study.

Name	Sequence	Description
BamPflaB-fwd	5'- CGGGATCCTGTCTGTCGCCTCTTG -3'	5' Flanking primer for flaB promoter
1055-Fwd	5'- CGCGGATCCAATGGGATTTAAAGGCTTTGCATGTTC -3'	5' Flanking primer for amplification of *lp6.6* locus
1055-Rev	5'- AAAACTGCAGGGGAGAGGGGTTTAAAAACCTCTTTC -3'	3' Flanking primer for amplification of *lp6.6* locus
1057-Fwd	5'- CGGGGTACCATCTAAATCTAACTTAACGGGAAATGTATTTCTAAC -3'	5' Flanking primer for amplification of *BB0323* locus
1057-Rev	5'- AAAACTGCAGACAGCTTTTTGAGCTTCTCTAAGATAAAC -3'	3' Flanking primer for amplification of *BB0323* locus
1063-Fwd	5'- AAAACTGCAGACAGCTTTTTGAGCTTCTCTAAGATAAAC -3'	5' SOE primer for creating BB0323P25:mRFPΔ4
1063-Rev	5'- TGAAGCGCATGAACTCCTTGATGACGTCTGGAGGCGTTTTGCATG -3'	3' SOE primer for creating BB0323P25:mRFPΔ4
1065-Fwd	5'- AGTCAATGAAAAAGGACGTCATCAAGGAGTTCATGCGCTTCAAGG -3'	5' SOE primer for creating lp6.6:mRFPΔ4
1065-Rev	5'- CATGAACTCCTTGATGACGTCCTTTTTCATTGACTTTGTCATAG -3'	3' SOE primer for creating lp6.6:mRFPΔ4

Protease Accessibility and Membrane Localization

Proteinase K accessibility and sucrose density gradient ultracentrifugation for membrane fractionation were performed as described previously (Schulze,2006).

Plasmolysis of B. burgdorferi *cells*

Two 1.0ml tubes of actively-growing cultures of *B. burgdorferi* strain B31-e2 that had been electrotransformed with one of four plasmids (pRJS1009, pRJS1014, pRJS1016, or pRJS1041) were spun down for 5 minutes and washed once with 1.0ml ice-cold PBS + 5mM $MgCl_2$. To one tube, the pellet was resuspended in 50.0µl of PBS-Mg. To the other, 50.0µl of plasmolysis buffer (15% sucrose, 25mM HEPES, pH 7.4, 20mM NaN_3) was added. Tubes were kept on ice for 10min and cells were visualized under epifluorescence microscopy using a Texas Red filter.

Chapter V: Use of Mislocalized Epitope-Tagged Mutants of *Borrelia burgdorferi* OspA to Search for an Outer Membrane Lipoprotein Transporter

Abstract

In *Borrelia burgdorferi*, the final step in surface exposure of a lipoprotein involves translocation across the outer membrane. Our understanding of this mechanism is incomplete as no putative transporters have been identified to date. Here, we take the first steps toward identifying candidate components that may be involved in this process. Deletion of residue Ser22 from the major *B. burgdorferi* surface lipoprotein OspA results in mislocalization of the protein to the inner leaflet of the outer membrane. Epitope-tagging of both this mutant and the wild-type protein allow comparisons to be made of protein-protein interactions involving OspA when it is present on opposing leaflets of the same membrane. We use coaffinity purification and introduce an application of **Mu**lti-**d**imensional **P**rotein **I**nteraction **T**echnology (MudPIT) analysis to survey the *Borrelia* outer membrane for components of a translocation complex.

Introduction

The presence of proteins on both sides of a bacterial membrane implies a mechanism for transport across that membrane. Many systems exist for the purposes of protein secretion across bacterial membranes. Outside of *Borrelia burgdorferi*, the presence of lipoproteins on the surface of Gram-negative cells is unusual, with only a handful

of examples known. These proteins are localized to the surface using established mechanisms, sometimes dedicated to the transport of a single known substrate. PulA uses a Type-II secretion apparatus for its secretion(Sandkvist,2001). SphB1 and NalP are lipoproteins from *Bordetella pertussis* and *Neisseria meningitidis* that use an autotransporter (Type-V) pathway for their placement on the cell surface (Coutte,2001;van Ulsen,2003). *Borrelia burgdorferi* has numerous lipoproteins on its surface, many of which are secreted to the surface by a default pathway using an as-of-yet-identified mechanism (Schulze,2006).

Searches of the *B. burgdorferi* genome for homologues of Type II secretion system components have failed to yield anything that might function analogously to the Pul secreton or other similar secretion machineries. Additionally, analyses of the primary sequences and crystal structures of these surface lipoproteins have not revealed the presence of autotransporter domains. We hypothesize that a novel mechanism dedicated to lipoprotein secretion exists in the *B. burgdorferi* OM.

A previous study from our laboratory showed that removal of single amino acid residues from the unstructured N-terminal tether of OspA was sufficient to prevent that lipoprotein from being transported to the cell surface (Schulze,2009). The addition of a C-terminal histidine-tag to OspA revealed that the entire protein is still transported to the cell surface whereas removal of Val21, Ser22/23, or Leu24 from the same OspA-His fusion inhibited OM translocation of the protein. Interestingly,

the epitope tag could be removed from OspA by *in situ* treatment of cells with proteinase K, whereas the remainder of the protein could not. This indicated the possibility that these individual residue deletions may result in the formation of a 'trapped' translocation state of the peptide.

As the mutation results in a phenotype that obviously impedes OM translocation, we took advantage of the C-terminal epitope tag to determine whether we could identify potential OspA binding partners by co-affinity immunoprecipitaion or Multi-dimensional Protein Identification Technology (MudPIT). MudPIT allows for the rapid examination of complex protein mixtures (i.e. OM preparations) through the use of a coupled high-efficiency liquid chromatography / tandem mass spectrometry process (Washburn,2001;Washburn,2004). Importantly, if the subsurface OspA constructs were truly trapped in an intermediate state of OM translocation, we might be able to identify components of a putative OM translocon through a side-by-side comparison of OspA-His and $OspA_{\Delta S22}$-His pull-downs.

Results

Co-affinity immunoprecipitation with epitope-tagged OspA

His-tagged OspA and $OspA_{\Delta S22}$ were previously demonstrated to be sorted to the *B. burgdorferi* cell surface and inner leaflet of the OM (OMIL), respectively. We expressed these proteins in the $OspA^-$ strain (B313) and then lysed the cells in the presence of 1% *n*-dodecyl-β-D-maltoside (DDM). We ran the contents of the cell

lysates over a cobalt affinity column to determine whether there were any stable interactions with periplasmic proteins resulting from the mislocalization of a surface lipoprotein in the OMIL.

As can be seen in the silver-stained gel of the various fractions (Fig. 20), the 100mM imidazole elution fractions appear relatively clean and contain only a few major visible bands. A ~25kD protein appears to be present in the 100mM elution for all four samples, suggestive of a contaminant. Outside of this band, however, very little protein is seen for the untagged OspA constructs. OspC, a surface lipoprotein whose expression is often variable and inconsistent during *in vitro* growth (Ramamoorthy,1998) is expressed in the strain containing OspA-His but not that of $OspA_{\Delta S22}$-His. Other minor bands of unknown nature appear in the elution fractions.

Identification of bound proteins

To verify the presence of OspC in the 100mM elution fraction of OspA-His, we performed Western blot analysis using a monoclonal antibody against OspC. As is evident from Fig. 21, OspC was confirmed to copurify to some degree with OspA-His. An antibody against OspA verified that the pull-downs were relatively specific for the epitope-tagged versions of the proteins. We also probed the elutions for the presence of p13, a *Borrelia* OM porin protein and IpLA7, a known IM-specific lipoprotein. As expected, a Western against IpLA7 showed that it was present only in

Figure 20. Co-affinity purification. This figure depicts a silver-stained gel loaded with samples from different washes and elutions for OspA, OspA-His, OspAΔS22, and OspAΔS22-His affinity purification on a cobalt resin column. A ~25kD band appears in the 100mM imidazole elution fractions for all five samples, indicative of a nonspecific (labeled N/S) contaminant. The variably expressed OspC is also evident in the strain expressing OspA-His but not OspAΔS22-His.

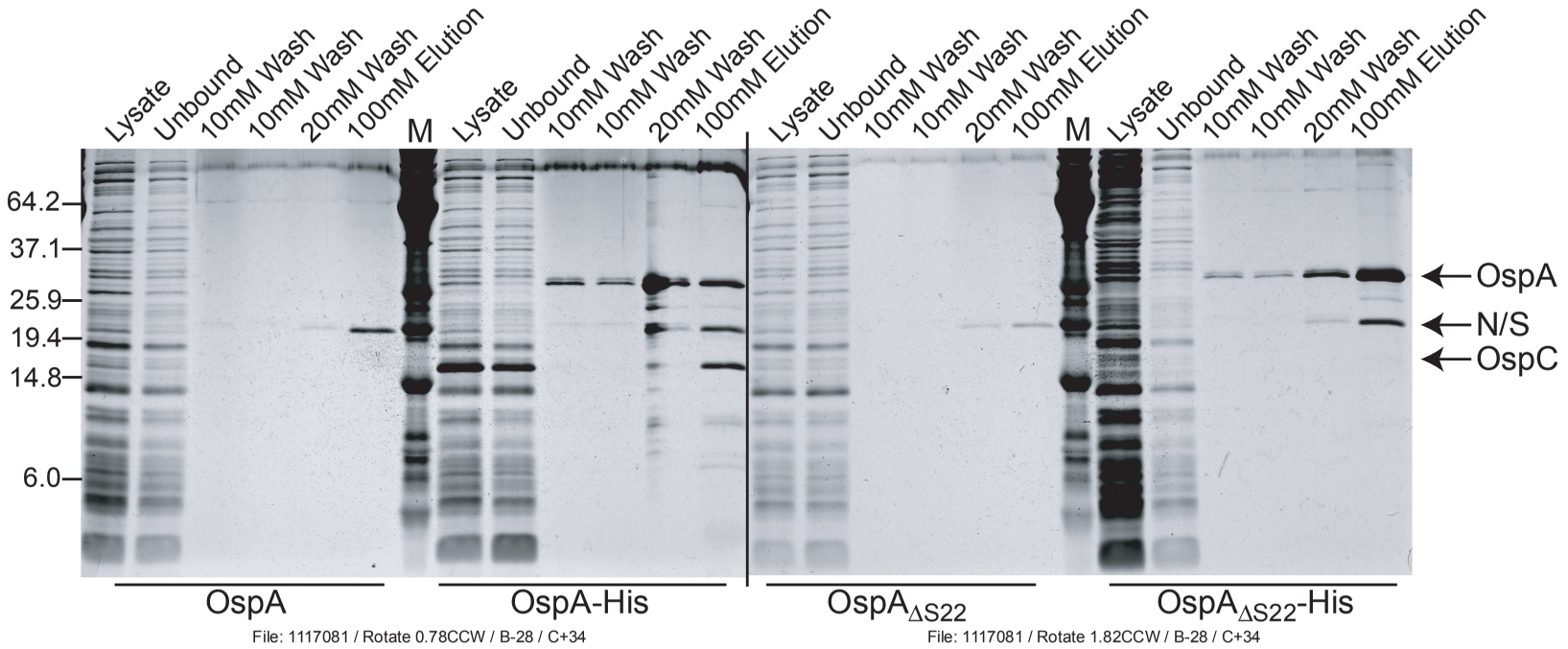
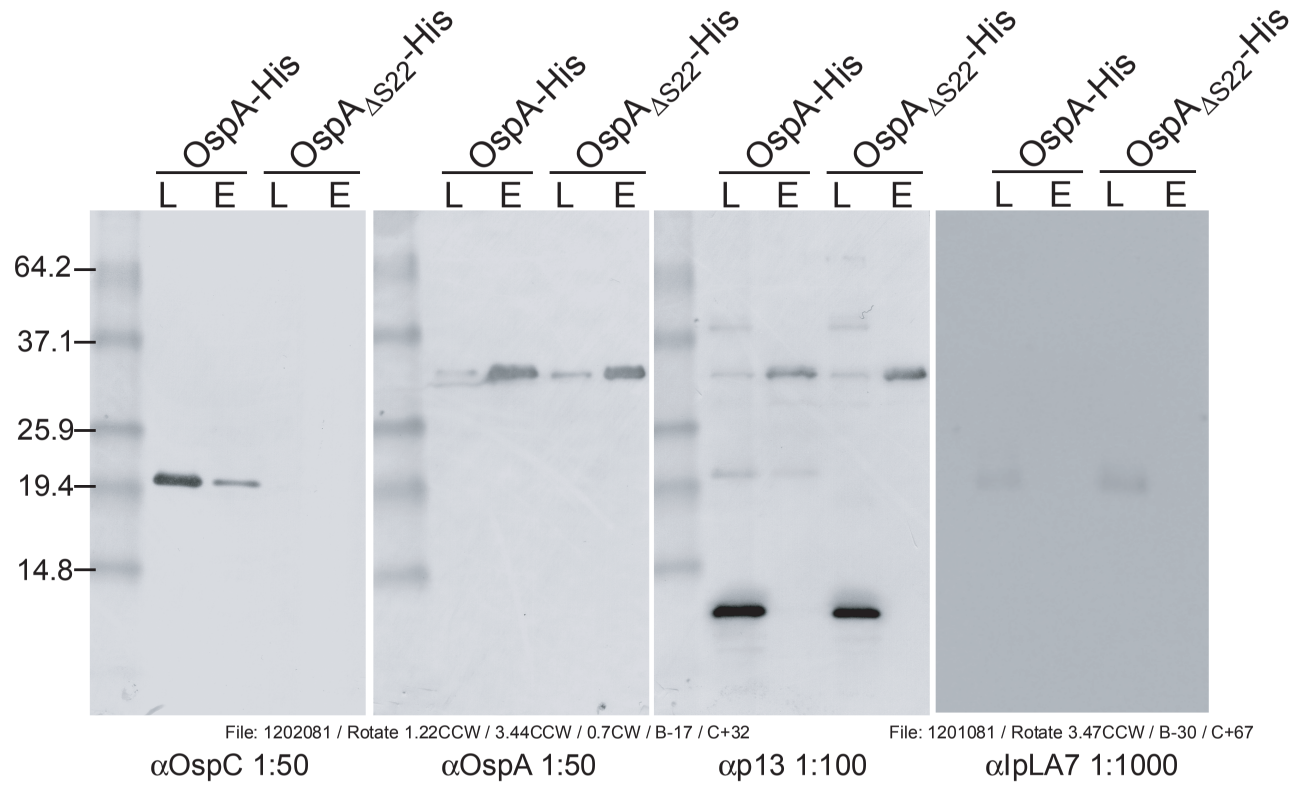

Lysate
Unbound
10mM Wash
10mM Wash
20mM Wash
100mM Elution
M
64.2
37.1
25.9
19.4
14.8
6.0
OspA
OspA-His
OspA$_{\Delta S22}$
OspA$_{\Delta S22}$-His
OspA
N/S
OspC
File: 1117081 / Rotate 0.78CCW / B-28 / C+34
File: 1117081 / Rotate 1.82CCW / B-28 / C+34

Figure 21. Western analysis of co-affinity purified fractions. We used antibodies against OspC, OspA, p13, and IpLA7 to preliminarily determine whether any of these were found in the 100mM elution fractions. This figure shows a comparison between the cell lysate (L) fraction and the 100mM elution (E) fraction. OspC is identified as the band seen in Figure 20 for the strain expressing OspA-His. OspA is enriched in the elution fraction, indicating that the His-tag pull-down was effective. Use of polyclonal p13 antisera showed cross-reactivity with both OspA and OspC. The prominent band indicates that the p13 porin is not found in the elution fraction. The same is true for the IM lipoprotein IpLA7, used here as a negative control.


OspA-His
OspA$_{\Delta S22}$-His
L E L E
64.2
37.1
25.9
19.4
14.8
File: 1202081 / Rotate 1.22CCW / 3.44CCW / 0.7CW / B-17 / C+32
File: 1201081 / Rotate 3.47CCW / B-30 / C+67
αOspC 1:50
αOspA 1:50
αp13 1:100
αIpLA7 1:1000

the flowthrough and likely played no part in interactions at the OM. The p13 protein used for antibody production was purified from an outer membrane protein preparation and may explain why the antibody shows reactivity to both OspA and OspC (Noppa,2001). The Western analysis reveals that p13 (the lower band) is not specifically pulled down by the epitope-tagged OspA.

We next used MudPIT analysis to determine the identity of the minor bands present in the sample. We show the thirty most abundant proteins from the samples in Tables 11 and 12. Table 11 indicates which peptides were identified by the analysis as well as the percentage of the total sample corresponded to that peptide. NSAF ratios are shown to indicate whether there was a noticeable increase or decrease in the presence of a given protein between surface exposed OspA-His and subsurface $OspA_{\Delta S22}$-His. As can be seen, many of these appear to be unlikely candidates for components of a putative OM translocon. Three proteins, CtpA, p66, and BB0028 are of special interest. The thirty peptides displayed in Tables 1 and 2 account for 96.98% and 87.44% of the spectra observed for runs of the OspA-His and $OspA_{\Delta S22}$-His samples, respectively.

Discussion

Here, we used epitope-tagged lipoproteins, determined from previous studies to be localized to opposite leaflets of the *Borrelia* OM, as a starting point in a search for a

Table 11. MudPIT NSAF data - 30 most abundant proteins

Locus	Description	OspA-His	OspA	OspAΔ22-His	OspAΔ22	Length	MW	pI	NSAF Ratio 22/A	NSAF Ratio A/22
OspA-His	**OspA-His {Borrelia burgdorferi B31}**	**71.85%**	**0.00%**	**60.59%**	**0.05%**	**285**	**30589**	**8.7**	**0.843289388**	1.185832544
BB_A15	**outer surface protein A (ospA) {Borrelia burgdorferi B31}**	**0.00%**	**0.03%**	**0.00%**	**0.00%**	**273**	**29367**	**8.7**		
BB_0151	N-acetylglucosamine-6-phosphate deacetylase (nagA)	4.05%	12.21%	6.94%	13.25%	401	44203	7	1.711858136	0.584160556
BB_0476	translation elongation factor TU (tuf)	0.14%	0.80%	0.53%	1.34%	401	44330	6.1	3.648480663	0.274086693
BB_0616	conserved hypothetical integral membrane protein	5.32%	1.91%	6.41%	2.48%	451	52050	8.3	1.203784434	0.830713516
BB_0649	heat shock protein (groEL)	0.07%	0.12%	0.02%	0.15%	545	58952	5.2	0.273239437	3.659793814
BB_0057	glyceraldehyde 3-phosphate dehydrogenase (gap)	0.17%	0.19%	0.00%	0.15%	335	36255	8		
BB_B19	outer surface protein C (ospC)	9.49%	0.00%	0.32%	0.06%	210	22341	8.3	0.033211848	30.10973676
BB_0560	heat shock protein 90 (htpG)	0.06%	0.00%	0.02%	0.05%	650	75424	6.9	0.273489933	3.656441718
BB_0478	ribosomal protein L3 (rplC)	0.00%	6.53%	0.75%	5.83%	206	22212	10		
BB_0147	flagellar filament 41 kDa core protein (flaB)	0.00%	0.54%	0.03%	0.35%	336	35765	5.7		
BB_0435	DNA gyrase, subunit A (gyrA)	0.00%	0.31%	0.12%	0.29%	810	91379	7.3		
BB_0388	DNA-directed RNA polymerase (rpoC)	0.00%	0.01%	0.02%	0.04%	1377	154657	8		
BB_0339	ribosomal protein L13 (rplM)	0.00%	0.25%	0.09%	0.40%	146	16694	10.1		
BB_0365	lipoprotein LA7	0.00%	0.52%	2.03%	0.67%	194	21866	5.8		
BB_0690	neutrophil activating protein (napA)	0.00%	0.19%	0.29%	0.14%	189	22414	5.5		
BB_0389	DNA-directed RNA polymerase (rpoB)	0.00%	0.02%	0.01%	0.06%	1155	129624	6.1		
BB_0359	carboxyl-terminal protease (ctp)	0.00%	0.04%	0.02%	0.04%	475	53542	8.3		
BB_0020	pyrophosphate--fructose 6-p 1-ptransferase (pfpB)	0.00%	0.02%	0.02%	0.06%	555	62477	6.6		
BB_0615	ribosomal protein S4 (rpsD)	4.45%	28.72%	8.22%	36.05%	209	24084	10.3	1.849889774	0.540572749
BB_0481	ribosomal protein L2 (rplB)	0.14%	2.18%	0.35%	2.66%	277	30591	10.5	2.530042918	0.395250212
BB_0405	hypothetical protein	0.10%	0.77%	0.17%	0.70%	203	22252	5.2	1.779643232	0.561910377
BB_0603	membrane-associated protein p66	0.85%	0.00%	0.44%	0.00%	618	68172	6.3	0.516911069	1.934568749
BB_0028	lipoprotein, putative	0.28%	0.00%	0.01%	0.00%	349	39705	8.8	0.02740714	36.48684211
BB_B29	PTS system, maltose and glucose-specific comp. (malX)	0.00%	0.05%	0.03%	0.04%	542	58938	8.6		
BB_0751	hypothetical protein	0.00%	0.03%	0.00%	0.14%	357	41901	6.5		
	Totals	96.98%		87.44%						

Table 12. MudPIT P/S/SC data - 30 most abundant proteins

		P	S	SC	P	S	SC	P	S	SC	P	S	SC
Locus	**Description**	**OspA-His**	**OspA-His**	**OspA-His**	**OspA**	**OspA**	**OspA**	**Δ22-His**	**Δ22-His**	**Δ22-His**	**OspAΔ22**	**OspAΔ22**	**OspAΔ22**
OspA-His	**OspA-His {Borrelia burgdorferi B31}**	34	1058	80.35				86	6521	92.63	1	2	6.32
BB_A15	**outer surface protein A (ospA) {Borrelia burgdorferi B31}**				1	1	8.42						
BB_0151	N-acetylglucosamine-6-phosphate deacetylase (nagA)	7	84	32.92	18	535	49.88	24	1051	72.57	36	823	63.09
BB_0476	translation elongation factor TU (tuf)	2	3	8.73	10	35	38.15	13	80	38.15	16	83	43.39
BB_0616	conserved hypothetical integral membrane protein	10	124	21.29	13	94	26.61	19	1091	42.35	17	173	30.38
BB_0649	heat shock protein (groEL)	2	2	8.81	3	7	9.72	1	4	5.32	7	13	17.98
BB_0057	glyceraldehyde 3-phosphate dehydrogenase (gap)	1	3	5.97	2	7	8.36				3	8	9.55
BB_B19	outer surface protein C (ospC)	11	103	58.1				4	25	27.62	1	2	7.14
BB_0560	heat shock protein 90 (htpG)	1	2	3.38				2	4	4	4	5	8
BB_0478	ribosomal protein L3 (rplC)				7	147	31.55	8	58	47.57	12	186	58.74
BB_0147	flagellar filament 41 kDa core protein (flaB)				8	20	27.68	2	4	8.93	6	18	29.46
BB_0435	DNA gyrase, subunit A (gyrA)				8	27	13.95	5	37	10.37	13	37	19.51
BB_0388	DNA-directed RNA polymerase (rpoC)				1	1	1.09	4	11	5.37	5	8	4.58
BB_0339	ribosomal protein L13 (rplM)				3	4	28.77	2	5	21.23	5	9	42.47
BB_0365	lipoprotein LA7				2	11	14.43	8	149	63.92	4	20	30.41
BB_0690	neutrophil activating protein (napA)				1	4	8.99	3	21	31.22	2	4	12.7
BB_0389	DNA-directed RNA polymerase (rpoB)				2	2	3.2	3	5	3.81	5	10	6.93
BB_0359	carboxyl-terminal protease (ctp)				2	2	4.63	1	4	3.37	2	3	5.89
BB_0020	pyrophosphate--fructose 6-p 1-ptransferase (pfpB)				1	1	3.42	4	4	16.04	3	5	9.37
BB_0615	ribosomal protein S4 (rpsD)	8	48	45.45	28	656	79.43	22	649	65.55	50	1167	86.6
BB_0481	ribosomal protein L2 (rplB)	1	2	5.05	8	66	36.46	8	37	35.74	22	114	57.4
BB_0405	hypothetical protein	1	1	10.84	4	17	21.18	3	13	24.14	5	22	23.65
BB_0603	membrane-associated protein p66	4	27	14.89				9	102	31.39			
BB_0028	lipoprotein, putative	2	5	8.6				1	1	5.44			
BB_B29	PTS system, maltose and glucose-specific comp. (malX)				2	3	5.35	2	7	6.27	2	3	5.35
BB_0751	hypothetical protein				1	1	2.52				4	8	18.21
		P	S	SC	P	S	SC	P	S	SC	P	S	SC

Legend: **P: Peptides S: Spectra SC: %Sequence Coverage**

putative OM translocation channel. Based on data from (Schulze,2009), we hypothesized that the mutant $OspA_{\Delta S22}$-His protein may be involved in a stalled translocation intermediate, perhaps stably interacting with components of a channel through which it would normally pass to the cell surface. We used coaffinity purification and the sensitive MudPIT analysis to identify proteins potentially involved in this transport process..

Certainly, the MudPIT results identify several proteins that are likely to be contaminants. BB0151 encodes an N-acetylglucosamine-6-phosphate deacetylase, an enzyme that plays a role in cell-wall biosynthesis. It contains several histidine-rich regions within its primary sequence, and because it shows up in the negative controls, we conclude that this is likely a contaminant. The same can be said for BB0616, a hypothetical protein with possible cation efflux pump properties. A close examination of its primary sequence reveals a site with the following primary sequence: HDHCHSHDHDHDHNHDH. It is therefore likely that the increased presence of this protein is due to binding to the cobalt column via this region. BB0615 is likely the non-specific band. It has a predicted molecular weight of approximately the same size and is present in abundance for all four samples. Unlike BB0151 and BB0616, it does not seem to have numerous histidines that might cause it to be pulled down as background. It is interesting to note that the ORFs *BB0615* (encoding a predicted integral membrane protein) and *BB0616* (encoding a predicted 30S ribosomal protein) are separated by 166 nucleotides on the *B. burgdorferi* linear chromosome,

suggesting that they might actually represent a single gene. Of the remaining proteins identified by the MudPIT screen, perhaps the three that deserve the most attention going forward are CtpA (BB0359), p66 (BB0603), and the hypothetical lipoprotein BB0028.

CtpA is a carboxy-terminal protease known to be closely linked with porin activity in *Borrelia*. It is required for the processing and maturation of the p13 porin and in its absence, the Oms28 porin protein is highly upregulated (Skare,1996;Noppa,2001;Ostberg,2004). It has also been shown to cleave the BB0323 lipoprotein, which we previously localized to the *Borrelia* IM (Chapter IV). Our MudPIT data shows that the subsurface $OspA_{\Delta S22}$-His protein but not the surface OspA-His protein pulls down CtpA. Examination of Table 2 reveals, however, that only a single trypsin fragment was identified in the $OspA_{\Delta S22}$-His sample and that CtpA is also seen in the non-epitope-tagged samples. Therefore, the associations seen here may be artifactual. Still, the close relationship CtpA has with OM porin activity in *Borrelia* must be further investigated.

Analysis of the p66 protein shows that it is a likely porin with the ability to bind to β_3-chain integrins on the surfaces of mammalian cells (Skare,1997;Cugini,2003;Coburn,1999). The adhesin function seems to be misleading considering p66 has only very small loops that extend outwards from the surface of the cell. Infectious *B. burgdorferi* contains a "rainforest" of lipoproteins on its

exterior that hinder the ability of antibodies from accessing smaller proteins (and surface loops like those found on p66) underneath (Bunikis,1995;Bunikis,1996;Bunikis,1999). Why a large protein with predicted porin activity would primarily act as an adhesin is unknown. An insertional inactivation of the *BB0603* gene results in decreased channel conductance (approximately 11nS) in planar lipid bilayer assays (Pinne,2007). The nature of the substrate for the p66 channel is unknown. The large conductance is suggestive that the pore is quite large in comparison to known *E. coli* OM porins that have conductances ranging from the picosiemens (pS) scale to ~2nS (Benz,1985;Pinne,2007). Could p66 have a role in the transport of lipoproteins to the surface? It would be interesting to examine the membrane topology of OspA (and other lipoproteins) in the Δp66 strain. Reports show that p66 can be pulled-down by formaldehyde cross-linking to OspA (Bunikis,1999). Interestingly, p66 is preferentially expressed during mammalian infection and is not seen in unfed ticks (Cugini,2003). Though this is the opposite of what is known about OspA expression, the finding that p66 might associate with this major surface lipoprotein should be further explored. Although our MudPIT data shows that p66 only represents 0.85% and 0.44% of the total peptides found in the OspA-His and $OspA_{\Delta S22}$-His fractions respectively (Table 11), it is not seen in the background samples. This indicates specificity of the interaction between OspA and p66.

BB0028 is a hypothetical lipoprotein of unknown function with homologues found only in other species of *Borrelia*. We previously analyzed the primary sequence of this lipoprotein and found that it is predicted to have a shorter N-terminal tether (perhaps as short as eight residues, with a sequence of CSSESIFS) than most other *B. burgdorferi* lipoproteins examined (Schulze,2009). Expression levels of the BB0028 mRNA transcript appear to be greatly elevated during CNS infection of non-human primates (Narasimhan,2003) and marginally elevated during growth of *Borrelia* under oxidative stress conditions (Hyde,2006). Aside from these two examples, nothing else is known about this lipoprotein. It is noteworthy that BB0028, like p66, specifically came down in the epitope-tagged samples but did not appear in the control samples.

In using MudPIT for this analysis, we are making an assumption that a stable interaction exists between either the surface OspA-His or subsurface $OspA_{\Delta S22}$-His protein with a component of a putative transporter. It is altogether feasible that mislocalized proteins are freely released into the OMIL and the cause for their mislocalization is a result of their inability to properly interact with a translocation complex. In such a scenario, the screen we use here would not reveal the nature of the transporter.

Regardless, the use of MudPIT for identifying potential OM-translocation complexes has demonstrated merit in that it was able to supplement known results (p66) and reveal proteins that might otherwise be overlooked (BB0028). It may be

disadvantageous, however, due to the sheer volume and complexity of the data it returns. One must be careful overinterpreting the results as it is an extremely sensitive assay, returning information that could never identified by other screens. In some cases, the data resolution is to within a single peptide fragment appearing in the spectra. At any rate, fine-tuning and careful control of the input samples may result in MudPIT proving to be a useful tool in the study of the OM architecture and protein transport mechanisms of *B. burgdorferi*. The treatment of OspA-His- and $OspA_{\Delta S22}$-His-expressing cells with various crosslinking agents prior to affinity purification may reveal additional interesting interactions not observed here.

Materials and Methods

Strains and Plasmids

Borrelia burgdorferi strain B313 has been described previously (Schulze,2006), as have the following plasmids pRJS0998 ($OspA_{wt}$), pRJS1203 ($OspA_{\Delta S22}$), pRJS1140 ($OspA_{\Delta S22}$-His), and pCSY6a-LinkerHis ($OspA_{wt}$-His).

Total Protein Prep

Four 250.0ml cultures of *Borrelia burgdorferi* strain B313 expressing OspA, OspA-His, $OspA_{\Delta S22}$, or $OspA_{\Delta S22}$-His were grown to a density of $5x10^7$ spirochetes/ml at 34°C + 5% CO_2. Cells were washed twice with PBS+5mM $MgCl_2$ and then resuspended in 20ml lysis buffer (50mM Tris, pH 7.4 / 400mM NaCl / 10mM imidazole / 1% *n*-dodecyl-β-D-maltoside (DDM) / 1mM PMSF). Cells were lysed in

a French pressure cell at 14,000psi (two passages/sample) and solubilize with rotation at 4°C for one hour. Unbroken cells/debris were removed by spinning samples for 15min at 10,000*g*. The supernatant was collected and used for metal affinity chromatography.

Metal Affinity Chromatography

The supernatant was applied to 2ml Talon® Metal Affinity Resin (Clontech) that was pre-washed 2x with binding buffer (50mM Tris, pH 7.4 / 400mM NaCl / 10mM imidazole / 0.1% DDM / 1mM PMSF). Binding was done for 1h at RT on a rotator. The resin was spun down at 700*g* for 3 min and flow-through was collected. The resin washed three times with 20ml binding buffer and resuspended in a final volume of 2ml binding buffer prior to being transferred to a gravity flow column. The resin was allowed to settle and buffer was drained. The column was washed twice with 5ml binding buffer (10mM imidazole) and proteins were eluted by treatment with 10ml binding buffer containing 20mM imidazole and then 100mM imidazole.

Analysis

Fractions were concentrated and the buffer was exchanged to Tris-HCl, pH 7.4 using an Amicon Ultra-15 centrifugal filter device (3kDa MW-cutoff). The protein concentration was determined using Bio-Rad DC protein assay (BSA standard). Samples were run on a 12.5% polyacrylamide gel and complexity was visualized by a

silver stain. Proteins were precipitated in trichloroacetic acid and submitted for MudPIT analysis.

MudPIT

TCA-precipiated proteins were denatured in 8M urea and treated with 10mM iodoacetamide (IAM) and 5mM *tris*(2-carboxyethyl)phosphine (TCEP) prior to overnight incubation with Endoproteinase Lys-C (0.1μg/μl) at 37°C. Samples were diluted to 2M urea with 100mM Tris-Hcl, pH 8.5 and $CaCl_2$ was added to 2mM prior to overnight trypsinization (0.1μg/μl) at 37°C. Samples were adjusted to 5% formic acid.

The tip of a triple-phase fused-silica microcapillary column (100μm i.d. x 365μm o.d. fused silica (Polymicro Technologies)) was pulled with a laser puller (Sutter Instrument Co. Model P-2000) and packed with 8cm of 5μm C_{18} reverse-phase (Aqua, Phenomenex), 3cm of 5μm strong cation exchange material (Partisphere SCX, Whatman), and another 1cm of 5μm C_{18} reverse phase. The column was washed with methanol for 10min and equilibrated in buffer A (5% ACN/0.1% Formic acid) for 30min.

The digested protein sample was spun down at 14,000rpm for 30min and pressure-loaded onto the column. The column was washed for 10min in buffer A and placed in-line with a LTQ linear ion trap mass spectrometer equipped with an electrospray

ionization source. With a flow rate of 200-300nl/min, a 10-step chromatography run (20hr) was carried out on the samples in buffer A, buffer B (80% CAN/0.1% formic acid), and buffer C (500mM ammonium acetate, 5% ACN, 0.1% formic acid), with the last two chromatography steps in a high salt wash with 100% Buffer C followed by the acetonitrile gradient to 100% Buffer B.

A 2.5kV voltage was applied distally to electrospray the eluting peptides. Full MS spectra were recorded on the peptides over a 400 to 1,600 *m*/*z* range, followed by five tandem mass (MS/MS) events sequentially generated in a data-dependent manner on the first to fifth most intense ions selected from the full MS spectrum (at 35% collisional energy).

Chapter VI: Discussion

The work presented here represents the very first insights into the process of lipoprotein secretion and sorting in *Borrelia burgdorferi*. We have demonstrated the existence of a pathway through which lipoproteins are funneled as they are targeted toward the outer surface of the cell. We clearly demonstrate that *B. burgdorferi* does not use the same sorting algorithm used by the Enterobacteriaceae or other Gram-negatives. Three lipoproteins have been shown by our laboratory (OppAIV, IpLA7, and BB0323) to likely reside in the inner membrane. The N-terminal four residues for each of these proteins are CVNE (OppAIV), CTSK (IpLA7), and CKTP (BB0323). From this data alone we are able to immediately rule out a '+2 rule' based on knowledge about three well-characterized surface-localized lipoproteins:

- OspD (CVHD) has a valine at +2 like OppAIV
- BBA66/p35 (CTID) has a threonine at +2 like IpLA7 (Hughes,2008)
- OspA (CKQN) has a lysine at +2 like BB0323.

Moreover, the addition of the N-terminal 31 residues of OppAIV to mRFP1 results in surface localization of the fluorescent reporter (Schulze,2006). We were able to show that negatively-charged residues can act as IM retention signals, but only in an appropriate context. We are not at this time able to determine the basis for this retention. Because the presence of negatively-charged residues may impact IM

release, we suggest that the initial stages of lipoprotein transport may be sequence-dependent in nature.

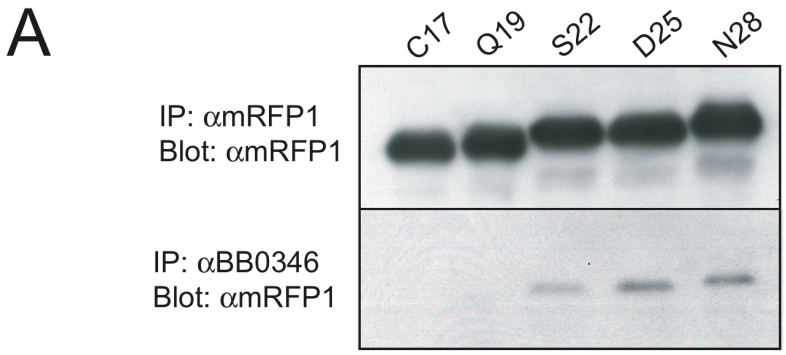
We show evidence that surface exposure may be the default pathway for lipoproteins in *Borrelia*, much the same way that the OM is the 'default' target of lipoproteins in *E. coli*. We also reveal a possible intriguing role for structural disorder in lipoprotein transport. Table 3 (p. 90) suggests that N-terminal disorder may be a phenomenon common to lipoproteins of all bacterial phyla. Though the N-termini of *B. burgdorferi* have very little in common sequence-wise, they all seem to have a tendency to be made up of residues typically found in unstructured domains. Removal of single residues from within this region (Val21-Ser22-Ser-23-Leu24 from OspA, for instance) or mutagenesis to prevent structural formation (VSSL → GGGG) all negatively impact OM translocation. Interestingly, the addition of an alanine residue at an alternative location within the tether was able to rescue the defect seen when Leu24 was removed. This suggests a sequence-independent mechanism for transport to the cell surface. Are lipoproteins of *Borrelia* required to adopt a translocation-competent conformation before they can be transported? What role would flexibility play in determining whether a protein might be secreted to the cell surface? These are questions that all should be investigated. We identified lp6.6 (BBA62) as a lipoprotein which resides in the inner leaflet of the OM (OMIL). A disorder prediction run on this peptide using the VSL2B algorithm suggests that it is

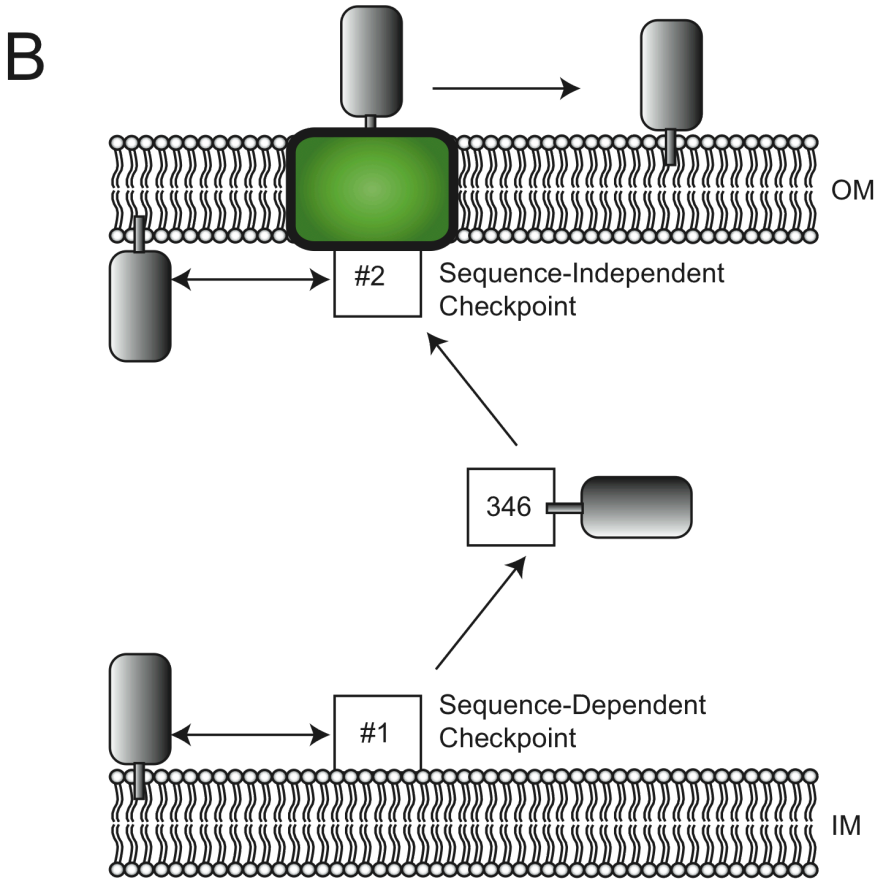
completely disordered (Schulze, 2009, submitted). Could this be the reason it is retained in the OMIL rather than transported to the surface?

With regard to the mechanism of transport between membranes, we know that *Borrelia* has homologues to the LolCDE components as well as a homologue to the periplasmic chaperone LolA. Do these proteins function analogously in *Borrelia*? Are the mechanisms of LolCDE avoidance related to interactions of negatively-charged side chains with membrane phospholipids (Hara,2003)? Do the LolCDE proteins even have the same function in *Borrelia*? These are questions that may be addressed in the near future. We have preliminary evidence that BB0346, the LolA homologue, interacts specifically with those lipoproteins that are transported to the surface. Using polyclonal antibodes to BB0346 for immunoprecipitation, we show in Fig. 22A that only OspA22-, OspA25-, and OspA28-mRFP, all identified in Chapter II to be surface-localized constructs, interact with and are co-immunoprecipitated by BB0346.

We think it is likely that *Borrelia* follows a Lol-like mechanism for IM release and periplasmic transport but then follows a different mechanism at the OM. Our model, as shown in Fig. 22B, suggests that lipoprotein N-termini are disordered and that this condition may be sufficient for transport to the cell surface, provided they are able to undergo a transition to a potential translocation-competent state (possibly due to binding by a periplasmic chaperone, see Fig. 15, p.88). In the event that this region is not stabilized in a correct conformation, then translocation across the OM can be

Figure 22. Role for *Borrelia* BB0346 and model for transport. (A) We performed two immunoprecipitations on whole cell lysates of *Borrelia burgdorferi* strain B31-e2 expressing one of five OspA:mRFP1 fusions. C17- and Q19-mRFP1 have been previously demonstrated to be localized exclusively to the *B. burgdorferi* IM (Schulze,2006). S22-, D25-, and N28-mRFP1 were previously localized in the same reference to the *B. burgdorferi* cell surface. Here, we show that IP with antibodies against mRFP1 shows the presence of each fusion protein in the five strains. IP with antibodies against the *B. burgdorferi* homologue of *E. coli* LolA (BB0346) reveals that only those fusions transported to the surface specifically interact with the putative chaperone. (B) A model for lipoprotein transport in *Borrelia*. At the IM, the presence of negatively-charged residues can retain lipoproteins at this step (checkpoint #1). If no retention signal is present, the lipoprotein is transported by the BB0346 chaperone to an unknown receptor in the OM. Here (checkpoint #2), sequence-independent signals such as tether flexibility/conformation may contribute to determining whether a lipoprotein is secreted to the cell surface or retained in the inner leaflet of the OM.


A
C17
Q19
S22
D25
N28
IP: αmRFP1
Blot: αmRFP1
IP: αBB0346
Blot: αmRFP1


B
OM
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impeded. If, alternatively, sequence-specific signals such as negative charge are present in the N-terminus, then release to the BB0346 chaperone from the IM might be prevented.

Further examination of the residues that are present at each position may provide hints into which residues play regulatory roles in lipoprotein transport. For instance, why are aspartic acid residues *never* found at the +3 position? Why instead are leucine residues so common? These types of questions can also be examined for lipoproteins of organisms orther than *Borrelia*. Why are prolines never found at the +2/+3 positions of *E. coli* lipoproteins, for example? See Table 6, p.93 for more examples.

In addition to revealing information about the sorting rules of *B. burgdorferi* lipoproteins, we demonstrate the potential advantages to using fluorescent or epitope-tagged reporters for lipoprotein localization studies. Chapters IV and V detail ways we might be able to employ mislocalized lipoproteins to i) identify novel subsurface retention signals (through the use of a FACS-based approach) and ii) identify novel components of a putative OM translocon (through an affinity co-purification approach).

It is possible that the results from these studies will have applications in understanding the biology and pathogenesis of other spirochetes, namely *Treponema*

pallidum and *Leptospira interrogans*. These two pathogens do not have the same large number of surface-exposed lipoproteins seen in *B. burgdorferi*; in fact, many of *T. pallidum*'s lipoproteins are thought to instead be anchored to the cytoplasmic membrane (Weigel,1994;Salazar,2002). The genetic intractability of treponemes has made it difficult to study lipoprotein biology as it relates to the pathogenesis of the organism. Perhaps the heterologous expression of *T. pallidum* lipoproteins in *Borrelia* would provide exciting new insights into the pathogenesis of the syphilis spirochete. Our laboratory has shown previously that surface lipoproteins of relapsing fever spirochetes can be displayed on the surface of *B. burgdorferi* (Zuckert,2004). It is not known whether surface transport of lipoproteins is a conserved feature amongst the spirochetes.

In summary, the work performed here provides new insights into an unexplored system of bacterial protein sorting and transport. Though bacterial lipoprotein localization studies have been ongoing for many years, it is quite clear that *B. burgdorferi* uses strikingly different mechanisms to accomplish its task of secreting lipoproteins to its cell surface.

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